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(54) **ANTIBODY FRAGMENT-POLYMER CONJUGATES**

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• C. DELGADO ET AL.: "Enhanced tumour specificity of an anti-carcinoembryonic antigen Fab' fragment by poly(ethylene glycol) (PEG) modification." BRITISH JOURNAL OF CANCER, vol. 73, no. 2, January 1996, pages 175-182, XP002084719 London, GB

Description

FIELD OF THE INVENTION

[0001] This application relates to the field of antibody fragments derivatized with polymers, and in particular to the use of such derivatization to increase the circulation half-lives of antibody fragment-polymer conjugates.

BACKGROUND

[0002] Modification of proteins with polyethylene glycol ("PEGylation") has the potential to increase residence time and reduce immunogenicity in vivo. For example, Knauf et al., J. Biol. Chem., 263: 15064-15070 (1988) reported a study of the pharmacodynamic behavior in rats of various polyoxylated glycerol and polyethylene glycol modified species of interleukin-2. Despite the known advantage of PEGylation, PEGylated proteins have not been widely exploited for clinical applications. In the case of antibody fragments, PEGylation has not been shown to extend serum half-life to useful levels. Delgado et al., Br. J. Cancer, 73: 175-182 (1996), Kitamura et al., Cancer Res., 51: 4310-4315 (1991), Kitamura et al., Biochem. Biophys. Res. Comm., 171: 1387-1394 (1990), and Pedley et al., Br. J. Cancer, 70: 1126-1130 (1994) reported studies characterizing blood clearance and tissue uptake of certain anti-tumor antigen antibodies or antibody fragments derivatized with low molecular weight (5 kD) PEG. Zapata et al., FASEB J., 9: A 1479 (1995) reported that low molecular weight (5 or 10 kD) PEG attached to a sulfhydryl group in the hinge region of a Fab' fragment reduced clearance compared to the parental Fab' molecule.

[0003] Interleukin-8 (IL-8) is neutrophil chemotactic peptide secreted by a variety of cells in response to inflammatory mediators (for a review see Hebert *et al.* Cancer Investigation 11(6):743 (1993)). IL-8 can play an important role in the pathogenesis of inflammatory disorders, such as adult respiratory distress syndrome (ARDS), septic shock, and multiple organ failure. Immune therapy for such inflammatory disorders can include treatment of an affected patient with anti-IL-8 antibodies.

[0004] Sticherling *et al.* (J. Immunol. 143:1628 (1989)) disclose the production and characterization of four monoclonal antibodies against IL-8. WO 92/04372, published March 19, 1992, discloses polyclonal antibodies which react with the receptor-interacting site of IL-8 and peptide analogs of IL-8, along with the use of such antibodies to prevent an inflammatory response in patients. St. John *et al.* (Chest 103:932 (1993)) review immune therapy for ARDS, septic shock, and multiple organ failure, including the potential therapeutic use of anti-IL-8 antibodies. Sekido *et al.* (Nature 365:654 (1993)) disclose the prevention of lung reperfusion injury in rabbits by a monoclonal antibody against IL-8. Mulligan *et al.* (J. Immunol. 150:5585 (1993)), disclose protective effects of a murine monoclonal antibody to human IL-8 in inflammatory lung injury in rats.

[0005] WO 95/23865 (International Application No. PCT/US95/02589 published September 8, 1995) demonstrates that anti-IL-8 monoclonal antibodies can be used therapeutically in the treatment of other inflammatory disorders, such as bacterial pneumonias and inflammatory bowel disease.

[0006] Anti-IL-8 antibodies are additionally useful as reagents for assaying IL-8. For example, Sticherling *et al.* (Arch. Dermatol. Res. 284:82 (1992)), disclose the use of anti-IL-8 monoclonal antibodies as reagents in immunohistochemical studies. Ko *et al.* (J. Immunol. Methods 149:227 (1992)) disclose the use of anti-IL-8 monoclonal antibodies as reagents in an enzyme-linked immunoabsorbent assay (ELISA) for IL-8.

SUMMARY OF THE INVENTION

[0007] The present invention provides a conjugate consisting essentially of one or more antibody fragments covalently attached to one or more polymer molecules as set out in claim 1.

BRIEF DESCRIPTION OF THE FIGURES

[0008]

Figure 1 is a graph depicting the blocking of IL-8 mediated elastase release from neutrophils by anti-IL-8 monoclonal antibody 5.12.14.

Figure 2 is a graph depicting the inhibition of ^{125}I-IL-8 binding to neutrophils by unlabeled IL-8.

Figure 3 demonstrates that a isotype matched negative control Fab (denoted as "4D5 Fab") does not inhibit the binding of ^{125}I-IL-8 to human neutrophils.

Figure 4 is a graph depicting the inhibition of binding of ^{125}I-IL-8 to human neutrophils by chimeric 5.12.14 Fab with an average IC_{50} of 1.6 nM.

Figure 5 is a graph depicting the inhibition of binding of ^{125}I-IL-8 to human neutrophils by chimeric 6G.4.25 Fab

with an average IC_{50} of 7.5 nM.

Figure 6 demonstrates the inhibition of human IL-8 mediated neutrophil chemotaxis by chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab.

Figure 7 demonstrates the relative abilities of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit rabbit IL-8 mediated neutrophil chemotaxis.

Figure 8 depicts the stimulation of elastase release from human neutrophils by various concentrations of human and rabbit IL-8. The relative extent of elastase release was quantitated by measurement of absorbance at 405 nm. The data represent mean ± SEM of triplicate samples.

Figure 9 is a graph depicting the ability of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit elastase release from human neutrophils stimulated by human IL-8. The results were normalized to reflect the percentage of elastase release elicited by 100 nM IL-8 alone. The data represent the mean ± SEM of three separate experiments performed on different days with different blood donors. IC_{50} values were calculated by four parameter fit.

Figure 10 is a graph depicting the relative abilities of chimeric 6G4.2.5 Fab and chimeric 5.12.14 Fab to inhibit elastase release from human neutrophils stimulated by rabbit IL-8. The results were normalized to reflect the percentage of elastase release elicited by 100 nM IL-8 alone. The data represent the mean ± SEM of three separate experiments performed on different days with different blood donors. IC_{50} values were calculated by four parameter fit.

Figures 11A-11J are a set of graphs depicting the following parameters in a rabbit ulcerative colitis model: Figure 11A depicts myeloperoxidase levels in tissue; Figure 11B depicts IL-8 levels in tissue; Figure 11C depicts colon weight; Figure 11D depicts gross inflammation; Figure 11E depicts edema; Figure 11F depicts extent of necrosis; Figure 11G depicts severity of necrosis; Figure 11H depicts neutrophil margination; Figure 11I depicts neutrophil infiltration; and Figure 11J depicts mononuclear infiltration.

Figure 12 is a graph depicting the effect of anti-IL-8 monoclonal antibody treatment on the number of neutrophils in bronchoalveolar lavage (BAL) fluid in animals infected with Streptococcus pneumoniae, Escherichia coli, or Pseudomonas aeruginosa. Treatment with 6G4.2.5 significantly reduced the number of neutrophils present in the BAL fluid compared to animals treated with isotype control mouse IgG (Figure 12).

Figure 13 depicts the DNA sequences (SEQ ID NOS: 1-6) of three primers designed for each of the light and heavy chains. Multiple primers were designed in order to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis for cloning the variable light and heavy regions of monoclonal antibody 5.12.14.

Figure 14 depicts the DNA sequences (SEQ ID NOS: 7-10) of one forward primer and one reverse primer for the 5.12.14 light chain variable region amplification.

Figure 15 depicts the DNA sequences (SEQ ID NOS: 11-15) of one forward primer and one reverse primer for the 5.12.14 heavy chain variable region amplification.

Figure 16 depicts the DNA sequence (SEQ ID NO: 16) and the amino acid sequence (SEQ ID NO: 17) of the 5.12.14 light chain variable region and partial murine constant light region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Important restriction sites are indicated in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids I to 109. The partial murine constant light region is amino acids 110 to 123 (in italics).

Figure 17 depicts the DNA sequence (SEQ ID NO: 18) and the amino acid sequence (SEQ ID NO: 19) of the 5.12.14 heavy chain variable region and partial murine constant heavy region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Important restriction sites are indicated in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 120. The partial murine constant heavy region is amino acids 121 to 130.

Figure 18 depicts the DNA sequences (SEQ ID NOS: 20-23) of amplification primers used to convert murine light and heavy chain constant region residues to their human equivalents.

Figure 19 depicts the DNA sequence (SEQ ID NO: 24) and the amino acid sequence (SEQ ID NO: 25) for the 5.12.14 light chain variable region and the human IgGI light chain constant region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 109. The human constant light region is amino acids 110 to 215.

Figures 20A-20B depict the DNA sequence (SEQ ID NO: 26) and the amino acid sequence (SEQ ID NO: 27) for the 5.12.14 heavy chain variable region and the heavy chain constant region of human IgG1. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids 1 to 120. The human constant heavy region is amino acids 121 to 229.

Figure 21 depicts the DNA sequences (SEQ ID NOS: 1-6) of three primers designed for each of the light and heavy

chains. Multiple primers were designed in order to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis for cloning the variable light and heavy regions of monoclonal antibody 6G4.2.5.

Figure 22 depicts the DNA sequences (SEQ ID NOS: 28-31) of one forward primer and one reverse primer for the 6G4.2.5 light chain variable region amplification.

Figure 23 depicts the DNA sequences (SEQ ID NOS: 32,33,11,15,14, and 13) of one forward primer and one reverse primer for the 6G4.2.5 heavy chain variable region amplification.

Figure 24 depicts the DNA sequence (SEQ ID NO: 34) and the amino acid sequence (SEQ ID NO: 35) of the 6G4.2.5 light chain variable region and partial murine constant light region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Useful cloning sites are in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable light region is amino acids 1 to 114. The partial murine constant light region is amino acids 115 to 131.

Figure 25 depicts the DNA sequence (SEQ ID NO: 36) and the amino acid sequence (SEQ ID NO: 37) of the 6G4.2.5 heavy chain variable region and partial murine constant heavy region. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). Useful cloning sites are in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids I to 122. The partial murine constant heavy region is amino acids 123 to 135.

Figure 26 depicts the DNA sequences (SEQ ID NOS: 38-40) of primers to convert the murine light chain and heavy chain constant regions to their human equivalents.

Figures 27A-27B depict the DNA sequence (SEQ ID NO: 41) and the amino acid sequence (SEQ ID NO: 42) for the chimeric 6G4.2.5 light chain. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids I to 114. The human constant heavy region is amino acids 115 to 220.

Figures 28A-28B depict the DNA sequence (SEQ ID NO: 43) and the amino acid sequence (SEQ ID NO: 44) for the chimeric 6G4.2.5 heavy chain. CDRs are indicated by either X-ray crystallography (underlined amino acids) or by Kabat sequence comparison (amino acids denoted with asterisk). The human constant region is denoted in italics. The signal peptide of STII is amino acids -23 to -1. The murine variable heavy region is amino acids I to 122. The human constant heavy region is amino acids 123 to 231.

Fig. 29 depicts an amino acid sequence alignment of murine 6G425 light chain variable domain (SEQ ID NO: 45), humanized 6G425 F(ab)-1 light chain variable domain (SEQ ID NO: 46), and human light chain κ1 consensus framework (SEQ ID NO: 47) amino acid sequences, and an amino acid sequence alignment of murine 6G425 heavy chain variable domain (SEQ ID NO: 48), humanized 6G425 F(ab)-1 heavy chain variable domain (SEQ ID NO: 49), and human IgG1 subgroup III heavy chain variable domain (SEQ ID NO: 50) amino acid sequences, used in the humanization of 6G425. Light chain CDRs are labeled L1, L2, L3; heavy chain CDRs are labeled H1, H2, and H3. = and + indicate CDR sequences as defined by X-ray crystallographic contacts and sequence hypervariability, respectively. # indicates a difference between the aligned sequences. Residue numbering is according to Kabat *et al.* Lower case lettering denotes the insertion of an amino acid residue relative to the humIII consensus sequence numbering.

Fig. 30 is a graph with three panels (A, B and C) depicting the ability of F(ab)-9 (humanized 6G4V11 Fab) to inhibit human wild type IL-8, human monomeric IL-8, and rhesus IL-8 mediated neutrophil chemotaxis, respectively. Panel A presents inhibition data for F(ab)-9 samples at concentrations of 0.06 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2nM human wild type IL-8. Panel B presents inhibition data for F(ab)-9 samples at concentrations of 6.25 nM, 12.5 nM, 25 nM, and 50 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 4 nM human monomeric IL-8 (denoted as "BD59" and as "monomeric IL-8"). Panel C presents inhibition data for F(ab)-9 samples at concentrations of 1 nM, 12.5 nM, 25 nM, and 50 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2 nM rhesus IL-8. In addition, all panels A, B an C each presents data for a no IL-8 buffer control sample (denoted as "Buffer") in the respective inhibition assay.

Fig. 31A depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V11 light chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 51), the humanized anti-IL-8 6G4.2.5V11 heavy chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 52), and a peptide linker in a C-terminal fusion with M 13 phage gene-III coat protein (SEQ ID NO: 53).

Fig. 31B depicts the nucleic acid sequence (SEQ ID NO: 54) and the translated amino acid sequence (SEQ ID NO: 51) of the humanized anti-IL-8 6G4.2.5V11 light chain in an N-terminal fusion with the STII leader peptide.

Fig. 31C depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V 19 light chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 51), and the humanized anti-IL-8 6G4.2.5V 19 heavy chain in an

N-terminal fusion with the STII leader peptide (SEQ ID NO: 55).

Fig. 32 is a three dimensional computer model of the humanized anti-IL-8 6G4.2.5V 11 antibody-Heavy chain CDR loops and variable domain regions appear in purple, and CDR-H3 side chain residues appear in yellow. Heavy chain constant domain regions appear in red. Light chain CDR loops and variable domain regions appear in off-white, and the Asn residue at amino acid position 35 (N35) in CDR L1 appears in green. Light chain constant domain regions appear in amber.

Fig. 33 is a Scatchard plot depicting the inhibition of ^{125}I-IL-8 binding to human neutrophils exhibited by intact murine 6G4.2.5 antibody (denoted 6G4 murine mAb), 6G4.2.5 murine-human chimera Fab (denoted 6G4 chimera), humanized 6G4.2.5 Fab versions 1 and 11 (denoted V1 and V11), and variant 6G4.2.5V11N35A Fab (denoted V11N35A).

Fig. 34 is a graph with four panels (A, B, C, and D) depicting the ability of 6G4.2.5V11N35A Fab to inhibit human wild type IL-8, human monomeric IL-8, rabbit IL-8, and rhesus IL-8 mediated neutrophil chemotaxis, respectively. Panel A presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "HuIL-8") sample, in the presence of 2 nM human wild type IL-8. Panel B presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "BD59") sample, in the presence of 2 nM human monomeric IL-8. Panel C presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "Rab IL-8") sample, in the presence of 2 nM rabbit IL-8. Panel D presents inhibition data for 6G4.2.5V11N35A Fab samples at concentrations of 0.5, 1, 2, 4, 8, 16, and 33 nM, for an intact 6G4.2.5 mAb sample at a concentration of 33 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 33 nM, and for a no antibody control (denoted "Rhe IL-8") sample, in the presence of 2 nM rhesus IL-8. In addition, panels B, C and D each presents data for human wild type IL-8 control (denoted "HuIL-8") samples at a concentration of 2 nM in the respective assay, and panels A, B, C, and D each presents data for a no IL-8 buffer control (denoted "Buffer") sample in the respective assay.

Fig. 35 depicts the amino acid sequences of the humanized anti-IL-8 6G4.2.5V11N35A light chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 56), the humanized anti-IL-8 6G4.2.5V11N35A heavy chain in an N-terminal fusion with the STII leader peptide (SEQ ID NO: 52), and the GCN4 leucine zipper peptide (SEQ ID NO: 57). The Ala residue (substituted for the wild type Asn residue) at amino acid position 35 in the 6G4.2.5V11N35A light chain appears in bold case. A putative pepsin cleavage site in the GCN4 leucine zipper sequence is underlined.

Fig. 36 depicts the DNA sequence (SEQ ID NO: 58) and the amino acid sequence (SEQ ID NO: 56) of the humanized anti-IL-8 6G4.2.5V11N35A light chain in an N-terminal fusion with the STII leader peptide. Complementarity determining regions L1, L2, and L3 are underlined

Figs. 37A-37B depict the DNA sequence (SEQ ID NO: 59) and the amino acid sequence (SEQ ID NO: 60) of the humanized anti-IL-8 6G4.2.5V11N35A heavy chain in an N-terminal fusion with the ST11 leader peptide and in a C-terminal fusion with the GCN4 leucine zipper sequence. Complementarity determining regions H1, H2, and H3 are underlined.

Fig. 38 is a Scatchard plot depicting the inhibition of ^{125}I-IL-8 binding to human neutrophils exhibited by 6G4.2.5V11N35A Fab (denoted Fab), 6G4.2.5V11N35A F(ab')$_2$ (denoted F(ab')$_2$), and human wild type IL-8 control (denoted IL-8).

Fig. 39 is a graph depicting a comparison of the wild type human IL-8 mediated neutrophil chemotaxis inhibition activities of the 6G4.2.5V11N35A F(ab')$_2$ and 6G4.2.5V11N35A Fab. Inhibition data are presented for 6G4.2.5V11N35A Fab samples (denoted "N35A Fab") and 6G4.2.5V11N35A F(ab')$_2$ samples (denoted N35A F (ab')$_2$) at concentrations of 0.3, 1, 3, 10, 30, and 100 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 100 nM, and for a no antibody control sample, in the presence of 2 nM human wild type IL-8. In addition, inhibition data are presented for no IL-8 buffer control samples (denoted "Buffer").

Fig. 40 is a graph depicting the ability of 6G4.2.5V11N35A F(ab')$_2$ to inhibit human monomeric IL-8, rhesus IL-8, and rabbit IL-8 mediated neutrophil chemotaxis. Human monomeric IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')$_2$ samples at concentrations of 0.3, 1, 3, and 10 nM, for an isotype control antibody (denoted as "4D5") sample at a concentration of 100 nM, and for a no antibody control sample (denoted as "BD59"), in the presence of human monomeric IL-8 (denoted as "BD59") at a concentration of 0.5 nM. Rhesus IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')$_2$ samples at concentrations of 0.3, 1, 3, and 10 nM, and for a no antibody control sample, in the presence of rhesus IL-8 at a concentration of 2 nM. Rabbit IL-8 mediated neutrophil chemotaxis data are presented for 6G4.2.5V11N35A F(ab')$_2$ samples at

concentrations of 0.3, 1, 3, and 10 nM, and for a no antibody control sample, in the presence of rabbit IL-8 at a concentration of 2 nM. In addition, inhibition data are presented for a no IL-8 buffer control sample (denoted as "Buffer") and for a 2 nM human wild type IL-8 (denoted as "HuIL-8").

Figs. 41A-41Q depict the nucleic acid sequence (SEQ ID NO: 61) of the p6G4V11N35A.F(ab')$_2$ vector.

Fig. 42 depicts the nucleic acid sequences of the stop template primer (SEQ ID NO: 63) and the NNS randomization primer (SEQ ID NO: 64) used for random mutagenesis of amino acid position 35 in variable light chain CDR-L1 of humanized antibody 6G4V11.

Fig. 43A is a table of data describing the frequencies of different phage display clones obtained from the randomization of amino acid position 35 in variable light chain CDR-L1 of humanized antibody 6G4V11.

Fig. 43B contains graphs of displacement curves depicting the inhibition of ^{125}I-IL-8 binding to neutrophils exhibited by the 6G4V11N35A, 6G4V11N35D, 6G4V11N35E and 6G4V11N35G Fab's.

Fig. 44 contains a graph depicting the typical kinetics of an anti-IL-8 antibody fragment (6G4V11N35A F(ab')$_2$) binding to IL-8. Fig. 44 also contains a table of data providing the equilibrium constant for 6G4V11N35A Fab binding to IL-8 (rate constants were not determined "ND"), and the equilibrium and rate constants for 6G4V11N35A F(ab')$_2$ and 6G4V11N35E Fab binding to IL-8.

Fig. 45 depicts the DNA sequence (SEQ ID NO: 65) and amino acid sequence (SEQ ID NO: 62) of the 6G4V11N35E light chain in an N-terminal fusion with the STII leader peptide. Complementarity determining regions L1, L2 and L3 are underlined.

Fig. 46 is a graph depicting the ability of 6G4V11N35E Fab to inhibit human IL-8 (dark columns) and rabbit IL-8 (light columns) mediated neutrophil chemotaxis. Data are presented for 6G4V11N35E Fab samples at concentrations of 0.4, 1.2, 3.7, 11 and 33 nM, and for an isotype control antibody (4D5) sample at a concentration of 100 nM, in the presence of 2 nM human IL-8 or 2 nM rabbit IL-8. In addition, inhibition data are presented for a no IL-8 buffer control sample (denoted "Buffer") and for human and rabbit IL-8 control samples (denoted "IL-8").

Fig. 47 depicts the DNA sequence of the sense (SEQ ID NO: 66) and anti-sense (SEQ ID NO: 67) strands of a PvuII-XhoI synthetic nucleotide encoding amino acids Leu4 to Phe29 of the 6G4V11N35A heavy chain.

Figs. 48A-48T depict the DNA sequence (SEQ ID NO: 68) of plasmid p6G4V11N35A.choSD9.

Fig. 49 contains graphs of displacement curves depicting the inhibition of ^{125}I-IL-8 binding to neutrophils exhibited by the full length IgG1 forms of variants 6G4V11N35A and 6G4V11N35E.

Figs. 50A-50B are graphs depicting the ability of full length 6G4V11N35A IgG1 and 6G4V11N35E IgG1 to inhibit human IL-8 (Fig. 50A) and rabbit IL-8 (Fig. 50B) mediated neutrophil chemotaxis.

Fig. 51 contains a graph depicting the typical kinetics of a full length anti-IL8 antibody (6G4V11N35A IgG1) binding to IL-8. Fig. 51 also contains a table of data providing the equilibrium and rate constants for full length murine 6G4.2.5 IgG2a, 6G4V11N35A IgG1 and 6G4V11N35E IgG1 binding to IL-8.

Fig. 52 contains graphs of displacement curves depicting the results of an unlabeled IL-8/^{125}I-IL-8 competition radioimmunoassay performed with full length 6G4V11N35A IgG1 and 6G4V11N35E IgG1.

Fig. 53 depicts the DNA sequence (SEQ ID NO: 69) and amino acid sequence (SEQ ID NO: 70) of the 6G4V11N35A Fab' heavy chain (6G4V11N35A Fab heavy chain modified to contain a cysteine residue in the hinge region).

Figs. 54A-54C contain graphs of displacement curves depicting the IL-8 binding and IC$_{50}$'s for PEG-maleimide modified 6G4V11N35A Fab' molecules.

Figs. 55A-55C are graphs depicting the ability of PEG-maleimide modified 6G4V11N35A Fab' molecules to inhibit human IL-8 and rabbit IL-8 mediated neutrophil chemotaxis.

Figs. 56A-56C are graphs depicting the ability of PEG-maleimide modified 6G4V11N35A Fab' molecules to inhibit IL-8 mediated release of β-glucuronidase from neutrophils.

Figs. 57A-57B contain graphs of displacement curves depicting the inhibition of ^{125}I-IL-8 binding to neutrophils exhibited by PEG-succinimide modified 6G4V11N35A Fab'$_2$ molecules.

Figs. 58A-58B are graphs depicting the ability of PEG-succinimide modified 6G4V 11N35A F(ab')$_2$ molecules to inhibit human IL-8 mediated neutrophil chemotaxis.

Figs. 59A-59B are graphs depicting the ability of PEG-succinimide modified 6G4V11N35A F(ab')$_2$ molecules to inhibit human IL-8 mediated release of β-glucuronidase from neutrophils.

Fig. 60 is a graph depicting the theoretical molecular weight (dotted bars) and effective size (solid bars) of PEG-maleimide modified 6G4V11N35A Fab' molecules as determined by SEC-HPLC.

Fig. 61 is an SDS-PAGE gel depicting the electrophoretic mobility of various PEG-maleimide modified 6G4V11N35A Fab' molecules.

Fig. 62 contains size exclusion chromatograms (SEC-HPLC) depicting the retention times and effective (hydrodynamic) sizes of various PEG-succinimide modified 6G4V11N35A F(ab')$_2$ molecules.

Fig. 63 is a graph depicting the theoretical molecular weight (open columns), effective size determined by SEC-HPLC (solid columns), and the actual molecular weight determined by SEC-light scattering (shaded columns) for various PEG-succinimide modified 6G4V11N35A F(ab')$_2$ molecules.

Fig. 64 is an SDS-PAGE gel depicting the electrophoretic mobility of various PEG-succinimide modified 6G4V11N35A F(ab')$_2$ molecules. From left to right, lane 1 contains unmodified F(ab')$_2$, lane 2 contains F(ab')$_2$ coupled to two 40 kD branched PEG-succinimide molecules (denoted "Br(2)-40kD(N)-F(ab')2"), lane 3 contains F(ab')$_2$ coupled to one 40 kD branched PEG-succinimide molecule (denoted "Br(l)-40kD-(N)-Fab'2"), lane 4 contains a mixture of F(ab')$_2$ coupled to four 20 kD linear PEG-succinimide molecules and F(ab')$_2$ coupled to five 20 kD linear'PEG-succinimide molecules (denoted "L(4+5)-20kD-(N)-Fab'2"), lane 5 contains F(ab')$_2$ coupled to one 20 kD linear PEG-succinimide molecule (denoted "L(1)-20kD-(N)-Fab'2"), and lane 6 contains molecular weight standards.

Fig. 65 contains graphs comparing the serum concentration vs. time profiles of various PEG-maleimide modified 6G4V11N35A Fab' molecules (upper graph) and various PEG-succinimide modified 6G4V11N35A F(ab')$_2$ molecules (lower graph) in rabbits. In the upper graph, "bran.(1)40K(s)Fab' " denotes 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule, "lin.(1)40K(s)Fab' " denotes 6G4V11N35A Fab' coupled to one 40 kD linear PEG-maleimide molecule, "lin.(1)30K(s)Fab' " denotes 6G4V11N35A Fab' coupled to one 30 kD linear PEG-maleimide molecule, "lin.(1)20K(s)Fab'" . denotes 6G4V11N35A Fab' coupled to one 20 kD linear PEG-maleimide molecule. In the lower graph, "bran.(2)40K(N)Fab'2" denotes 6G4V11N35A F(ab')$_2$ coupled to two 40 kD branched PEG-succinimide molecules, "bran.(1)40K(N)Fab'2" denotes 6G4V11N35A F(ab')$_2$ coupled to one 40 kD branched PEG-succinimide molecule, and "Fab'2" denotes unmodified 6G4V11N35A F(ab')$_2$. In both graphs. "IgG" denotes a full length IgG1 equivalent of the human-murine chimeric anti-rabbit IL-8 Fab described in Example F below.

Fig. 66 contains graphs comparing the serum concentration vs. time profiles of 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule (denoted as "bran.(1)40K(s)Fab'"), 6G4V11N35A F(ab')$_2$ coupled to one 40 kD branched PEG-succinimide molecule (denoted as "bran.(1)40K(N)Fab'2"), unmodified 6G4V11N35A F (ab')$_2$ (denoted as "Fab'2"), unmodified 6G4V11N35A Fab' (denoted as "Fab'"), and a full length IgGl (denoted as "IgG") equivalent of the human-murine chimeric anti-rabbit IL-8 Fab described in Example F below.

Fig. 67 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one 40 kD branched PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on gross weight of entire lung in an ARDS rabbit model.

Fig. 68 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one branched 40 kD PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on BAL total leukocyte (light columns) and polymorphonuclear cell (dark columns) counts in an ARDS rabbit model. Untreated (no therapeutics) control animal data is denoted as "Control".

Fig. 69 is a graph depicting the effect of 6G4V11N35A Fab' coupled to one branched 40 kD PEG-maleimide molecule (denoted as "PEG 40 Kd") and murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5 (full length IgG2a) (denoted as "6G4.2.5") on PaO2/FiO2 ratio at 24 hours-post treatment (light columns) and 48 hours post-treatment (dark columns) in an ARDS rabbit model. Untreated (no therapeutics) control animal data is denoted as "Control".

DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. DEFINITIONS

[0009] In general, the following words or phrases have the indicated definition when used in the description, examples, and claims.

[0010] "Polymerase chain reaction" or "PCR" refers to a procedure or technique in which minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers can coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis *et al.*, Cold Spring Harbor Symp. Quant. Biol. 51:263 (1987); Erlich, ed., PCR Technology (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

[0011] "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

[0012] "Native antibodies and immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons,

composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains (Clothia *et al.*, J. Mol. Biol. 186:651 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. U.S.A. 82:4592 (1985)).

[0013] The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.*, Sequences of Proteins of Immunological Interest, Fifth Edition, National Institute of Health, Bethesda, MD (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

[0014] Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0015] "Fv" is the minimum antibody fragment which contains a complete antigen-recognition and - binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species (scFv), one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site. For a review of scFv see Pluckthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

[0016] The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0017] The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (k) and lambda (l), based on the amino acid sequences of their constant domains.

[0018] Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these can be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, y, and p, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

[0019] The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies) and antibody compositions with polyepitopic specificity.

[0020] "Antibody fragment", and all grammatical variants thereof, as used herein are defined as a portion of an intact antibody comprising the antigen binding site or variable region of the intact antibody, wherein the portion is free of the constant heavy chain domains (i.e. CH2, CH3, and CH4, depending on antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include Fab, Fab', Fab'-SH, $F(ab')_2$, and Fv fragments; diabodies; any antibody fragment that is a polypeptide having a primary structure consisting of one uninterrupted sequence of contiguous amino acid residues (referred to herein as a "single-chain antibody fragment" or "single chain polypeptide"), including without limitation (1)single-chain Fv (scFv) molecules (2)single chain polypeptides containing only one light chain var-

iable domain, or a fragment thereof that contains the three CDRs of the light chain variable domain, without an associated heavy chain moiety and (3)single chain polypeptides containing only one heavy chain variable region, or a fragment thereof containing the three CDRs of the heavy chain variable region, without an associated light chain moiety; and multispecific or multivalent structures formed from antibody fragments. In an antibody fragment comprising one or more heavy chains, the heavy chain(s) can contain any constant domain sequence (e.g. CH1 in the IgG isotype) found in a non-Fc region of an intact antibody, and/or can contain . any hinge region sequence found in an intact antibody, and/or can contain a leucine zipper sequence fused to or situated in the hinge region sequence or the constant domain sequence of the heavy chain(s). Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney et al., J. Immunol., 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

[0021] Unless specifically indicated to the contrary, the term "conjugate" as described and claimed herein is defined as a heterogeneous molecule formed by the covalent attachment of one or more antibody fragment(s) to one or more polymer molecule(s), wherein the heterogeneous molecule is water soluble, i.e. soluble in physiological fluids such as blood, and wherein the heterogeneous molecule is free of any structured aggregate. In the context of the foregoing definition, the term "structured aggregate" refers to (1) any aggregate of molecules in aqueous solution having a spheroid or spheroid shell structure, such that the heterogeneous molecule is not in a micelle or other emulsion structure, and is not anchored to a lipid bilayer, vesicle or liposome; and (2) any aggregate of molecules in solid or insolubilized form, such as a chromatography bead matrix, that does not release the heterogeneous molecule into solution upon contact with an aqueous phase. Accordingly, the term "conjugate" as defined herein encompasses the aforementioned heterogeneous molecule in a precipitate, sediment, bioerodible matrix or other solid capable of releasing the heterogeneous molecule into aqueous solution upon hydration of the solid.

[0022] Unless specifically indicated to the contrary, the terms "polymer", "polymer molecule", "nonproteinaceous polymer", and "nonproteinaceous polymer molecule" are used interchangeably and are defined as a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is contained in the group consisting of alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), leucine (Leu), methionine (Met), asparagine (Asn), proline (Pro), glutamine (Gln), arginine (Arg), serine (Ser), threonine (Thr), valine (Val), tryptophan (Trp), and tyrosine (Tyr) residues.

[0023] The term "monoclonal antibody" (mAb) as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each mAb is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they can be synthesized by hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature*, 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U. S. Patent No. 4,816,567 to Cabilly *et al.*). The "monoclonal antibodies" also include clones of antigen-recognition and binding-site containing antibody fragments (Fv clones) isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991), for example.

[0024] The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-IL-8 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired biological activity. (See, e.g., U.S. Pat. No. 4,816,567 to Cabilly *et al.*; Mage and Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp. 79-97 (Marcel Dekker, Inc., New York, 1987).)

[0025] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly *et al.*, supra; Morrison *et al.*, Proc. Natl. Acad. Sci. U.S.A. 81:6851 (1984)).

[0026] "Humanized" forms of non-human (e.g., murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized

antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and maximize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al.*, Nature 321:522 (1986); Reichmann *et al.*, Nature 332:323 (1988); and Presta, Curr. Op. Struct. Biol. 2:593 (1992).

[0027] "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented.

[0028] "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal herein is human.

[0029] As used herein, protein, peptide and polypeptide are used interchangeably to denote an amino acid polymer or a set of two or more interacting or bound amino acid polymers.

[0030] As used herein, the term "inflammatory disorders" refers to pathological states resulting in inflammation, typically caused by neutrophil chemotaxis. Examples of such disorders include inflammatory skin diseases including psoriasis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); ischemic reperfusion; adult respiratory distress syndrome; dermatitis; meningitis; encephalitis; uveitis; autoimmune diseases such as rheumatoid arthritis, Sjorgen's syndrome, vasculitis; diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder, multiple organ injury syndrome secondary to septicaemia or trauma; alcoholic hepatitis, bacterial pneumonia, antigen-antibody complex mediated diseases; inflammations of the lung, including pleurisy, alveolitis, vasculitis, pneumonia, chronic bronchitis, bronchiectasis, and cystic fibrosis; etc. The preferred indications are bacterial pneumonia and inflammatory bowel disease such as ulcerative colitis.

[0031] The terms "hydrodynamic size", "apparent size", "apparent molecular weight", "effective size" and "effective molecular weight" of a molecule are used synonymously herein refer to the size of a molecule as determined by comparison to a standard curve produced with globular protein molecular weight standards in a size exclusion chromatography system, wherein the standard curve is created by mapping the actual molecular weight of each standard against its elution time observed in the size exclusion chromatography system. Thus, the apparent size of a test molecule is derived by using the molecule's elution time to extrapolate a putative molecular weight from the standard curve. Preferably, the molecular weight standards used to create the standard curve are selected such that the apparent size of the test molecule falls within the linear portion of the standard curve.

II. MODES FOR CARRYING OUT THE INVENTION

[0032] The invention arises from the surprising and unexpected discovery that antibody fragment-polymer conjugates having an effective or apparent size significantly greater than the antibody fragment-polymer conjugates described in the art confers an increase in serum half-life, an increase in mean residence time in circulation (MRT), and/or a decrease in serum clearance rate over underivatized antibody fragment which far exceed the modest changes in such biological property or properties obtained with the art-known antibody fragment-polymer conjugates. The present inventors have determined for the first time that increasing the effective size of an antibody fragment to at least about 500,000 D, or increasing the effective size of an antibody fragment by at least about 8 fold over the effective size of the parental antibody fragment, or derivatizing an antibody fragment with a polymer of at least about 20,000 D in molecular weight, yields a molecule with a commercially useful pharmacokinetic profile. The greatly extended serum half-life, extended MRT, and/or reduced serum clearance rate of the conjugates of the invention makes such conjugates viable alternatives to intact antibodies used for therapeutic treatment of many disease indications. Antibody fragments provide significant advantages over intact antibodies, notably the fact that recombinant antibody fragments can be made in bacterial cell expression systems. Bacterial cell expression systems provide several advantages over mammalian cell expression systems, including reduced time and cost at both the research and development and manufacturing stages of a product.

[0033] Humanization of the 6G4.2.5 murine anti-rabbit IL-8 monoclonal antibody ("6G4.2.5") is described in WO 95/23865 (PCT/US95/02589 published September 8, 1995). The hybridoma producing antibody 6G4.2.5 was deposited on September 28, 1994 with the American Type Culture Collection and assigned ATCC Accession No. HB 11722 as described in the Examples below.

[0034] It will be understood that in the context of this Section (II) and all subsections thereof, every reference to "an antibody fragment" or "the antibody fragment" contained in a conjugate shall be a reference to one or more antibody fragment(s) in the conjugate (consistent with the definition of the term "conjugate" set forth in Section (I) above), except where the number of antibody fragment(s) in the conjugate is expressly indicated. It will be understood that in the context of this Section (II) and all subsections thereof, every reference to "a polymer", "a polymer molecule", "the polymer", or "the polymer molecule" contained in a conjugate shall be a reference to one or more polymer molecule (s) in the conjugate (consistent with the definition of the term "conjugate" set forth in Section (I) above), except where the number of polymer molecule(s) in the conjugate is expressly indicated.

1. LARGE EFFECTIVE SIZE ANTIBODY FRAGMENT-POLYMER CONJUGATES

[0035] The antibody fragment may be covalently attached to a polymer to form a conjugate having an effective or apparent size of at least about 500,000 Daltons (D). The antibody fragment may be covalently attached to a polymer to form a conjugate having an apparent size that is at least about 8 fold greater than the apparent size of the parental antibody fragment. The antibody fragment may be covalently attached to a polymer of at least about 20,000 D in molecular weight (MW). It will be appreciated that the unexpectedly and surprisingly large increase in antibody fragment serum half-life, increase in MRT, and/or decrease in serum clearance rate can be achieved by using any type of polymer or number of polymer molecules which will provide the conjugate with an effective size of at least about 500,000 D, or by using any type of polymer or number of polymer molecules which will provide the conjugate with an effective size that is at least about 8 fold greater than the effective size of the parental antibody fragment, or by using any type or number of polymers wherein each polymer molecule is at least about 20,000 D in M W. Thus, the invention is not dependent on the use of any particular polymer or molar ratio of polymer to antibody fragment in the conjugate.

[0036] In addition, the beneficial aspects of the invention extend to antibody fragments without regard to antigen specificity. Although variations from antibody to antibody are to be expected, the antigen specificity of a given antibody will not substantially impair the extraordinary improvement in serum half-life, MRT, and/or serum clearance rate for antibody fragments thereof that can be obtained by derivatizing the antibody fragments as taught herein.

[0037] The conjugate may have an effective size of at least about 500,000 D, or at least about 800,000 D, or at least about 900,000 D, or at least about 1,000,000 D, or at least about 1,200,000 D, or at least about 1,400,000 D, or at least about 1,500,000 D, or at least about 1,800,000 D, or at least about 2,000,000 D, or at least about 2,500,000 D.

[0038] The conjugate may have an effective size of at or about 500,000 D to at or about 10,000,000 D, or an effective size of at or about 500,000 D to at or about 8,000,000 D, or an effective size of at or about 500,000 D to at or about 5,000,000 D, or an effective size of at or about 500,000 D to at or about 4,000,000 D, or an effective size of at or about 500,000 D to at or about 3,000,000 D, or an effective size of at or about 500,000 D to at or about 2,500,000 D, or an effective size of at or about 500,000 D to at or about 2,000,000 D, or an effective size of at or about 500,000 D to at or about 1,800,000 D, or an effective size of at or about 500,000 D to at or about 1,600,000 D, or an effective size of at or about 500,000 D to at or about 1,500,000 D, or an effective size of at or about 500,000 D to at or about 1,000,000 D.

[0039] The conjugate may have an effective size of at or about 800,000 D to at or about 10,000,000 D, or an effective size of at or about 800,000 D to at or about 8,000,000 D, or an effective size of at or about 800,000 D to at or about 5,000,000 D, or an effective size of at or about 800,000 D to at or about 4,000,000 D, or an effective size of at or about 800,000 D to at or about 3,000,000 D, or an effective size of at or about 800,000 D to at or about 2,500,000 D, or an effective size of at or about 800,000 D to at or about 2,000,000 D, or an effective size of at or about 800,000 D to at or about 1,800,000 D, or an effective size of at or about 800,000 D to at or about 1,600,000 D, or an effective size of at or about 800,000 D to at or about 1,500,000 D, or an effective size of at or about 800,000 D to at or about 1,000,000 D.

[0040] The conjugate may have an effective size of at or about 900,000 D to at or about 10,000,000 D, or an effective size of at or about 900,000 D to at or about 8,000,000 D, or an effective size of at or about 900,000 D to at or about 5,000,000 D, or an effective size of at or about 900,000 D to at or about 4,000,000 D, or an effective size of at or about 900,000 D to at or about 3,000,000 D, or an effective size of at or about 900,000 D to at or about 2,500,000 D, or an effective size of at or about 900,000 D to at or about 2,000,000 D, or an effective size of at or about 900,000 D to at or about 1,800,000 D, or an effective size of at or about 900,000 D to at or about 1,600,000 D, or an effective size of at or about 900,000 D to at or about 1,500,000 D.

[0041] The conjugate may have an effective size of at or about 1,000,000 D to at or about 10,000,000 D, or an effective size of at or about 1,000,000 D to at or about 8,000,000 D, or an effective size of at or about 1,000,000 D to at or about 5,000,000 D, or an effective size of at or about 1,000,000 D to at or about 4,000,000 D, or an effective size of at or about 1,000,000 D to at or about 3,000,000 D, or an effective size of at or about 1,000,000 D to at or about 2,500,000 D, or an effective size of at or about 1,000,000 D to at or about 2,000,000 D, or an effective size of at or about 1,000,000 D to at or about 1,800,000 D, or an effective size of at or about 1,000,000 D to at or about 1,600,000 D, or an effective size of at or about 1,000,000 D to at or about 1,500,000 D.

[0042] The conjugate may have an effective size that is at least about 8 fold greater, or at least about 10 fold greater,

or at least about 12 fold greater, or at least about 15 fold greater, or at least about 18 fold greater, or at least about 20 fold greater, or at least about 25 fold greater, or at least about 28 fold greater, or at least about 30 fold greater, or at least about 40 fold greater, than the effective size of the parental antibody fragment.

[0043] The conjugate may have an effective size that is about 8 fold to about 100 fold greater, or is about 8 fold to about 80 fold greater, or is about 8 fold to about 50 fold greater, or is about 8 fold to about 40 fold greater, or is about 8 fold to about 30 fold greater; or is about 8 fold to about 28 fold greater, or is about 8 fold to about 25 fold greater, or is about 8 fold to about 20 fold greater, or is about 8 fold to about 18 fold greater, or is about 8 fold to about 15 fold greater, than the effective size of the parental antibody fragment.

[0044] The conjugate may have an effective size that is about 12 fold to about 100 fold greater, or is about 12 fold to about 80 fold greater, or is about 12 fold to about 50 fold greater, or is about 12 fold to about 40 fold greater, or is about 12 fold to about 30 fold greater, or is about 12 fold to about 28 fold greater, or is about 12 fold to about 25 fold greater, or is about 12 fold to about 20 fold greater, or is about 12 fold to about 18 fold greater, or is about 12 fold to about 15 fold greater, than the effective size of the parental antibody fragment.

[0045] The conjugate may have an effective size that is about 15 fold to about 100 fold greater, or is about 15 fold to about 80 fold greater, or is about 15 fold to about 50 fold greater, or is about 15 fold to about 40 fold greater, or is about 15 fold to about 30 fold greater, or is about 15 fold to about 28 fold greater, or is about 15 fold to about 25 fold greater, or is about 15 fold to about 20 fold greater, or is about 15 fold to about 18 fold greater, than the effective size of the parental antibody fragment.

[0046] The conjugate may have an effective size that is about 18 fold to about 100 fold greater, or is about 18 fold to about 80 fold greater, or is about 18 fold to about 50 fold greater, or is about 18 fold to about 40 fold greater, or is about 18 fold to about 30 fold greater, or is about 18 fold to about 28 fold greater, or is about 18 fold to about 25 fold greater, or is about 18 fold to about 20 fold greater, than the effective size of the parental antibody fragment.

[0047] The conjugate may have an effective size that is about 20 fold to about 100 fold greater, or is about 20 fold to about 80 fold greater, or is about 20 fold to about 50 fold greater, or is about 20 fold to about 40 fold greater, or is about 20 fold to about 30 fold greater, or is about 20 fold to about 28 fold greater, or is about 20 fold to about 25 fold greater, than the effective size of the parental antibody fragment.

[0048] The conjugate may have an effective size that is about 25 fold to about 100 fold greater, or is about 25 fold to about 80 fold greater, or is about 25 fold to about 50 fold greater, or is about 25 fold to about 40 fold greater, or is about 25 fold to about 30 fold greater, or is about 25 fold to about 28 fold greater, than the effective size of the parental antibody fragment.

[0049] The conjugate may have an effective size that is about 28 fold to about 100 fold greater, or is about 28 fold to about 80 fold greater, or is about 28 fold to about 50 fold greater, or is about 28 fold to about 40 fold greater, or is about 28 fold to about 30 fold greater, than the effective size of the parental antibody fragment.

[0050] The conjugate may have an effective size that is about 30 fold to about 100 fold greater, or is about 30 fold to about 80 fold greater, or is about 30 fold to about 50 fold greater, or is about 30 fold to about 40 fold greater, than the effective size of the parental antibody fragment.

[0051] The conjugate may have an effective size that is about 40 fold to about 100 fold greater, or is about 40 fold to about 80 fold greater, or is about 40 fold to about 50 fold greater, than the effective size of the parental antibody fragment.

[0052] The antibody fragment may be covalently attached to at least one polymer having an actual M W of at least about 20,000 D.

[0053] The antibody fragment may be covalently attached to at least one polymer having an actual MW of at least about 30,000 D.

[0054] The antibody fragment may be covalently attached to at least one polymer having an actual MW of at least about 40,000 D.

[0055] The antibody fragment may be covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D.

[0056] The antibody fragment may be covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D.

[0057] The antibody fragment may be covalently attached to at least one polymer having an actual MW that is at or about 20,000 D to at or about 70,000 D, or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D.

[0058] The antibody fragment may be covalently attached to at least one polymer having an actual M W that is at or about 20,000 D to at or about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D.

[0059] The antibody fragment may be covalently attached to at least one polymer having an actual M W that is at or

about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D.

[0060] In one aspect, the invention encompasses a conjugate having any molar ratio of polymer to antibody fragment that endows the conjugate with an apparent size in the desired range as taught herein. The apparent size of the conjugate will depend in part upon the size and shape of the polymer used, the size and shape of the antibody fragment used, the number of polymer molecules attached to the antibody fragment, and the location of such attachment site (s) on the antibody fragment. These parameters can easily be identified and maximized to obtain the a conjugate with the desired apparent size for any type of antibody fragment, polymer and linkage system.

[0061] In another aspect, the invention encompasses a conjugate with a polymer to antibody fragment molar ratio of no more than about 10:1, or no more than about 5:1, or no more than about 4:1, or no more than about 3:1, or no more than about 2:1, or no more than 1:1.

[0062] In yet another aspect, the invention encompasses a conjugate wherein the antibody fragment is attached to about 10 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the conjugate contains an antibody fragment attached to about 5 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the conjugate contains an antibody fragment attached to about 4 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In a further embodiment, the conjugate contains an antibody fragment attached to about 3 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the conjugate contains an antibody fragment attached to about 2 or fewer polymer molecules, each polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is a conjugate containing an antibody fragment attached to a single polymer molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

[0063] In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

[0064] In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than I polymer molecule.

[0065] In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 70,000 D, or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than I polymer molecule.

[0066] In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

[0067] In still another aspect, the invention encompasses a conjugate wherein every polymer molecule in the conjugate has a molecular weight that is at or about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D, and wherein the conjugate contains no more than about 10 polymer molecules, or no more than about 5 polymer molecules, or no more than about 4 polymer molecules, or no more than about 3 polymer molecules, or no more than about 2 polymer molecules, or no more than 1 polymer molecule.

[0068] It is believed that the serum half-life, MRT and/or serum clearance rate of any antibody fragment can be greatly improved by derivatizing the antibody fragment with polymer as taught herein. In one embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv and $F(ab')_2$.

[0069] In one embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide

bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0070] In a further embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than I polymer molecule and the polymer is coupled to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0071] In a further embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at least about 20,000 D in molecular weight, or at least about 30,000 D in molecular weight, or at least about 40,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0072] In another embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0073] In another embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0074] In another embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0075] In another embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0076] In another embodiment, the conjugate contains a $F(ab')_2$ antibody fragment attached to no more than about 2 polymer molecules, wherein every polymer molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein every polymer molecule in the conjugate is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0077] In yet another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at least about 20,000 D in molecular weight, or at least about 30,000 D in molecular weight, or at least about 40,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0078] In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than I polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight,

wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0079] In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0080] In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0081] In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0082] In another embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than I polymer molecule, wherein the polymer molecule is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein the polymer molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0083] Although any type of polymer is contemplated for use in constructing the conjugates of the invention, including the polymers and chemical linkage systems described in Section (II)(I)(b) below, polyethylene glycol (PEG) polymers are preferred for use herein.

[0084] In one embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W of at least about 20,000 D.

[0085] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW of at least about 30,000 D.

[0086] In yet another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W of at least about 40,000 D.

[0087] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W that is at or about 20,000 D to at or about 300,000 D, or is at or about 30,000 D to at or about 300,000 D, or is at or about 40,000 D to at or about 300,000 D.

[0088] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W that is at or about 20,000 D to at or about 100,000 D, or is at or about 30,000 D to at or about 100,000 D, or is at or about 40,000 D to at or about 100,000 D.

[0089] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W that is at or about 20,000 D to at or about 70,000 D, or is at or about 30,000 D to at or about 70,000 D, or is at or about 40,000 D to at or about 70,000 D.

[0090] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual M W that is at or about 20,000 D to at or about 50,000 D, or is at or about 30,000 D to at or about 50,000 D, or is at or about 40,000 D to at or about 50,000 D.

[0091] In another embodiment, the conjugate is an antibody fragment covalently attached to at least one PEG having an actual MW that is at or about 20,000 D to at or about 40,000 D, or is at or about 30,000 D to at or about 40,000 D.

[0092] In another aspect, the invention encompasses a conjugate with a PEG to antibody fragment molar ratio of no more than about 10:1, or no more than about 5:1, or no more than about 4:1, or no more than about 3:1, or no more than about 2:1, or no more than 1:1.

[0093] In yet another aspect, the invention encompasses a conjugate wherein the antibody fragment is attached to

about 10 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the conjugate contains an antibody fragment attached to about 5 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the conjugate contains an antibody fragment attached to about 4 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In a further embodiment, the conjugate contains an antibody fragment attached to about 3 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the conjugate contains an antibody fragment attached to about 2 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is a conjugate containing an antibody fragment attached to a single PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

[0094] In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than I PEG molecule.

[0095] In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than I PEG molecule.

[0096] In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than I PEG molecule.

[0097] In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0098] In another aspect, the invention encompasses a conjugate wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than I PEG molecule.

[0099] In still another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and $F(ab')_2$, wherein the antibody fragment is attached to about 10 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In another embodiment, the foregoing conjugate contains an antibody fragment attached to about 5 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In still another embodiment, the foregoing conjugate contains an antibody fragment attached to about 4 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In a further embodiment, the foregoing conjugate contains an antibody fragment attached to about 3 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. In an additional embodiment, the foregoing conjugate contains an antibody fragment attached to about 2 or fewer PEG molecules, each PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. Also provided herein is the foregoing conjugate that contains an antibody fragment attached to a single PEG molecule having a molecular weight of at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D.

[0100] In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and $F(ab')_2$, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or

about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0101] In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')$_2$ wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0102] In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')$_2$, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0103] In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')$_2$, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0104] In another aspect, the invention encompasses a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', Fab'-SH and F(ab')$_2$, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, and wherein the conjugate contains no more than about 10 PEG molecules, or no more than about 5 PEG molecules, or no more than about 4 PEG molecules, or no more than about 3 PEG molecules, or no more than about 2 PEG molecules, or no more than 1 PEG molecule.

[0105] In yet another preferred embodiment, the conjugate contains a F(ab')$_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at least about 20,000D in molecular weight, or at least about 30,000D in molecular weight, or at least about 40,000D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0106] In another preferred embodiment, the conjugate contains a F(ab')$_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0107] In another preferred embodiment, the conjugate contains a F(ab')$_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0108] In another preferred embodiment, the conjugate contains a F(ab')$_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by sub-

stituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0109] In another preferred embodiment, the conjugate contains a $F(ab')_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0110] In another preferred embodiment, the conjugate contains a $F(ab')_2$ antibody fragment derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at or about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than about 2 PEG molecules, and wherein every PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0111] In still another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at least about 20,000 D in molecular weight, or at least about 30,000 in molecular weight, or at least about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0112] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 300,000 D in molecular weight, or is at or about 30,000 D to at or about 300,000 D in molecular weight, or is at or about 40,000 D to at or about 300,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0113] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 100,000 D in molecular weight, or is at or about 30,000 D to at or about 100,000 D in molecular weight, or is at or about 40,000 D to at or about 100,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0114] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 70,000 D in molecular weight, or is at or about 30,000 D to at or about 70,000 D in molecular weight, or is at or about 40,000 D to at or about 70,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0115] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 50,000 D in molecular weight, or is at or about 30,000 D to at or about 50,000 D in molecular weight, or is at or about 40,000 D to at or about 50,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0116] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is derivatized with PEG, wherein every PEG molecule in the conjugate is at or about 20,000 D to at or about 40,000 D in molecular weight, or is at or about 30,000 D to at

or about 40,000 D in molecular weight, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is attached to a cysteine residue in the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains, wherein the disulfide bridge is avoided by substituting another amino acid, such as serine, for the corresponding cysteine residue in the opposite chain.

[0117] It will be appreciated that all of the above-described embodiments of the invention utilizing PEG polymers include conjugates wherein the PEG polymer(s) is (are) linear or branched. In a preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and at least about 40,000 D in molecular weight. In a particularly surprising and unexpected finding, the inventors discovered that the foregoing conjugate exhibits a serum half-life, MRT and serum clearance rate approaching that of full length antibody as shown in Example X below.

[0118] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 300,000 D.

[0119] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 100,000 D.

[0120] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 70,000 D.

[0121] In another preferred embodiment, the conjugate contains an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, and wherein the PEG molecule is branched and has a molecular weight that is at or about 40,000 D to at or about 50,000 D.

[0122] In another preferred embodiment, the invention provides a conjugate containing an antibody fragment selected from the group consisting of Fab, Fab', and Fab'-SH, wherein the antibody fragment is attached to no more than 1 PEG molecule, wherein the PEG molecule is branched and at least 40,000D in molecular weight, and the PEG molecule is anached to the hinge region of the antibody fragment.

[0123] In one aspect, the invention provides any of the above-described conjugates wherein the conjugate contains no more than one antibody fragment. Additionally provided herein is any of the above-described conjugates wherein the conjugate contains one or more antibody fragment(s) covalently linked to one or more polymer molecule(s), such as conjugates containing two or more antibody fragments covalently linked together by polymer molecule(s). In one embodiment, a polymer molecule is used to link together two antibody fragments to form a dumbbell-shaped structure. Also encompassed herein are conjugates formed by more than two antibody fragments joined by polymer molecule (s) to form a rosette or other shapes. The antibody fragments in such structures can be of the same or different fragment type and can have the same antigen specificity or have different antigen specificities. Such structures can be made by using a polymer molecule derivatized with multiple functional groups permitting the direct attachment, or the attachment by means of bi- or multi-functional linkers, of two or more antibody fragments to the polymer backbone.

[0124] In another aspect, the invention encompasses any of the above-described conjugates utilizing an antibody fragment comprising an antigen recognition site that binds to rabbit IL-8 and/or human IL-8.

a. Production of Antibody Fragments

[0125] Antibody fragments can be produced by any method known in the art. Generally, an antibody fragment is derived from a parental intact antibody. The parental antibody can be generated by raising polyclonal sera against the desired antigen by multiple subcutaneous (sc) or intraperitoneal (ip) injections of antigen and an adjuvant, such as monophosphoryl lipid A (MPL)/trehalose dicrynomycolate (TDM) (Ribi Immunochem. Research, Inc., Hamilton, MT), at multiple sites. Two weeks later the animals are boosted. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer. Animals are boosted until titer plateaus. Sera are harvested from animals, and polyclonal antibodies are isolated from sera by conventional immunoglobulin purification procedures, such as protein A-Sepharose chromatography, hydroxylapatite chromatography, gel filtration, dialysis, or antigen affinity chromatography. The desired antibody fragments can be generated from purified polyclonal antibody preparations by conventional enzymatic methods, e.g. $F(ab')_2$ fragments are produced by pepsin cleavage of intact antibody, and Fab fragments are produced by briefly digesting intact antibody with papain.

[0126] Alternatively, antibody fragments are derived from monoclonal antibodies generated against the desired antigen. Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

[0127] In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that

will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

[0128] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[0129] Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

[0130] Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

[0131] The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al., Anal. Biochem.*, 107:220 (1980).

[0132] After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

[0133] The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0134] DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of antibody-encoding DNA include Skerra et al., Curr. Opinion in Immunol., 5: 256 (1993) and Pluckthun, Immunol. Revs., 130: 151 (1992).

[0135] In a preferred embodiment, the antibody fragment is derived from a humanized antibody. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. It will be appreciated that variable domain sequences obtained from any non-human animal phage display library-derived Fv clone or from any non-human animal hybridoma-derived antibody clone provided as described herein can serve as the "import" variable domain used in the construction of the humanized antibodies of the invention. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature*, **321**: 522 (1986); Riechmann *et al., Nature*, **332**: 323 (1988); Verhoeyen *et al., Science*, **239**: 1534 (1988)), by substituting non-human animal, e.g. rodent, CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (Cabilly *et al., supra*), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in non-human animal, e.g. rodent, antibodies.

[0136] The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a non-human animal, e.g. rodent, antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the non-human animal is then accepted as the human framework (FR) for the humanized antibody (Sims *et al., J. Immunol.*, **151**: 2296 (1993); Chothia and Lesk, *J. Mol. Biol.*, **196**: 901 (1987)). Another method uses a particular framework derived from the consensus sequence of all

human antibodies of a particular subgroup light or heavy chains. The same framework can be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad Sci USA,* **89**: 4285 (1992); Presta *et al., J. Immunol.,* **151**: 2623 (1993)). It is also important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind to its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[0137] In addition, antibody fragments for use herein can be derived from human monoclonal antibodies. Human monoclonal antibodies against the antigen of interest can be made by the hybridoma method. Human myeloma and mouse-human heteromyeloma cell lines for the production of human monoclonal antibodies have been described, for example, by Kozbor *J. Immunol.,* **133**: 3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications,* pp. 51-63 (Marcel Dekker, Inc., New York, 1987); and *Boemer et al., J. Immunol.,* **147**: 86 (1991).

[0138] It is now possible to produce transgenic animals (e.g. mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al., Proc. Natl. Acad Sci USA,* **90**: 2551 (1993); Jakobovits *et al., Nature,* **362**: 255 (1993); Bruggermann *et al., Year in Immunol.,* *7*: 33 (1993).

[0139] Alternatively, phage display technology (McCafferty et al., Nature **348**:552 (1990)) can be used to produce human antibodies and antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g., Johnson et al., Current Opinion in Structural Biology **3**:564 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature **352**:624 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. **222**:581 (1991), or Griffith et al., EMBO J. **12**:725 (1993).

[0140] In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks et al., Bio/Technol. 10:779 (1992)). In this method, the affinity of "primary" human antibodies obtained by phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires has been described by Waterhouse et al., Nucl. Acids Res. **21**:2265 (1993).

[0141] Gene shuffling can also be used to derive human antibodies from non-human, e.g. rodent, antibodies, where the human antibody has similar affinities and specificities to the starting non-human antibody. According to this method, which is also called "epitope imprinting", either the heavy or light chain variable region of a non-human antibody fragment obtained by phage display techniques as described above is replaced with a repertoire of human V domain genes, creating a population of non-human chain/human chain scFv or Fab chimeras. Selection with antigen results in isolation of a non-human chain/human chain chimeric scFv or Fab wherein the human chain restores the antigen binding site destroyed upon removal of the corresponding non-human chain in the primary phage display clone, i.e. the epitope governs (imprints) the choice of the human chain partner. When the process is repeated in order to replace the remaining non-human chain, a human antibody is obtained (see PCT WO 93/06213 published April 1, 1993). Unlike traditional humanization of non-human antibodies by CDR grafting, this technique provides completely human antibodies, which have no FR or CDR residues of non-human origin.

[0142] The invention also encompasses the use of bispecific and heteroconjugate antibody fragments having specificities for at least two different antigens. Bispecific and heteroconjugate antibodies can be prepared as full length antibodies or as antibody fragments (e.g. $F(ab')_2$ bispecific antibody fragments). Antibody fragments having more than two valencies (e.g. trivalent or higher valency antibody fragments) are also contemplated for use herein. Bispecific antibodies, heteroconjugate antibodies, and multi-valent antibodies can be prepared as described in Section (II)(3)(C) below.

[0143] As described above, DNA encoding the monoclonal antibody or antibody fragment of interest can be isolated from its hybridoma or phage display clone of origin, and then manipulated to create humanized and/or affinity matured constructs, In addition, known techniques can be employed to introduce an amino acid residue or residues into any desired location on the polypeptide backbone of the antibody fragment, e.g. a cysteine residue placed in the hinge region of the heavy chain, thereby providing a site for specific attachment of polymer molecule(s). In one embodiment, the native cysteine residue in either the light or heavy chain of the antibody fragment that would ordinarily form the disulfide bridge linking the light and heavy chains is substituted with another amino acid, such as serine, in order to leave the partner cysteine residue in the opposite chain with a free suflhydryl for specific attachment of polymer molecule.

[0144] Upon construction of the desired antibody or antibody fragment-encoding clone, the clone can be used for recombinant production of the antibody fragment as described in Section (II)(4) below. Finally, the antibody or antibody fragment product can be recovered from host cell culture and purified as described in Section (II)(4)(F) below. In the case of embodiments utilizing an antibody fragment engineered to lack a cysteine residue that ordinarily forms the disulfide bridge between the light and heavy chains as described above, preferred recombinant production systems include bacterial expression and product recovery procedures utilizing the low pH osmotic shock method described in the "Alternative Fab'-SH Purification" section of Example T below. If a full length antibody is produced, the desired antibody fragment can be obtained therefrom by subjecting the intact antibody to enzymatic digestion according to known methods, e.g. as described in Section (II)(4)(G) below.

b. Construction of Antibody Fragment-Polymer Conjugates

[0145] The antibody fragment-polymer conjugates of the invention can be made by derivatizing the desired antibody fragment with an inert polymer. It will be appreciated that any inert polymer which provides the conjugate with the desired apparent size or which has the selected actual MW as taught herein is suitable for use in constructing the antibody fragment-polymer conjugates of the invention.

[0146] Many inert polymers are suitable for use in pharmaceuticals. See, e.g., Davis et al., Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, pp.441-451 (1980). In all embodiments of the invention, a non-proteinaceous polymer is used. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i. e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are also useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyoxyalkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrins, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. Preferably, the conjugate exhibits a water solubility of at least about 0.01 mg/ml, and more preferably at least about 0.1 mg/ml, and still more preferably at least about I mg/ml. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if the conjugate is intended to be administered by such routes.

[0147] In one embodiment, the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to maximize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or ion exchange chromatography to recover substantially homogenous derivatives. In other embodiments, the polymer contains two or more reactive groups for the purpose of linking multiple antibody fragments to the polymer backbone. Again, gel filtration or ion exchange chromatography can be used to recover the desired derivative in substantially homogeneous form.

[0148] The molecular weight of the polymer can range up to about 500,000 D, and preferably is at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. The molecular weight chosen can depend upon the effective

size of the conjugate to be achieved, the nature (e.g. structure, such as linear or branched) of the polymer, and the degree of derivatization, i.e. the number of polymer molecules per antibody fragment, and the polymer attachment site or sites on the antibody fragment.

[0149] The polymer can be covalently linked to the antibody fragment through a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid residues of the antibody fragment to be linked. However, it is also within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the antibody fragment, or vice versa.

[0150] In addition to the cysteine residue described in claim 1, the covalent crosslinking site on the antibody fragment may include the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the antibody fragment without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, activated succinimidyl esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylcloroformate or P-nitrophenylctoroformate activated PEG.) Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide. Sulfhydryl groups are derivatized by coupling to maleimido-substituted PEG (e.g. alkoxy-PEG amine plus sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) as described in WO 97/10847 publistied March 27, 1997, or PEG-maleimide commercially available from Shearwater Polymers, Inc., Huntsville, AL). Alternatively, free amino groups on the antibody fragment (e.g. epsilon amino groups on lysine residues) can be thiolated with 2-imino-thiolane (Traut's reagent) and then coupled to maleimide-containing derivatives of PEG as described in Pedley et al., Br. J. Cancer, 70: 1126-1130 (1994).

[0151] The polymer will bear a group which is directly reactive with an amino acid side chain, or the Nor C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

[0152] "Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

[0153] The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the antibody fragment, the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular antibody fragment derivatization sites chosen. In general, the conjugate contains from 1 to about 10 polymer molecules, but greater numbers of polymer molecules attached to the antibody fragments of the invention are also contemplated. The desired amount of derivatization is easily achieved by using an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the level of polymer substitution of the conjugates is determined by size exclusion chromatography or other means known in the art.

[0154] The polymer, e.g. PEG, is cross-linked to the antibody fragment by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG.

[0155] Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, Anal Biochem. 131, 25-33 [1983]) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, J. Polym. Sci. Polym. Chem. Ed. 22, 341-52 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred. In another preferred embodiment, maleimido-activated PEG is used for coupling to free thiols on the antibody fragment.

[0156] Functionalized PEG polymers to modify the antibody fragments of the invention are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymeth-

ylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

[0157] The conjugates of this invention are separated from the unreacted starting materials by gel filtration or ion exchange HPLC. Heterologous species of the conjugates are purified from one another in the same fashion.

[0158] The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (e.g. containing one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids. In one embodiment, species with difference levels of PEGylation are resolved according to the methods described in WO 96/34015 (International Application No. PCT/US96/05550 published October 31, 1996).

[0159] In a preferred embodiment, the conjugate is generated by utilizing the derivatization and purification methods described in Section (T) of the Examples below.

[0160] In one aspect, the invention provides any of the above-described conjugates formed by its component parts, i.e. one or more antibody fragment(s) covalently attached to one or more polymer molecule(s), without any extraneous matter in the covalent molecular structure of the conjugate.

c. Other Derivatives of Large Effective Size Conjugates

[0161] In another aspect, any of the above-described conjugates can be modified to contain one or more component (s) in addition to the antibody fragment component(s) and polymer component(s) that form the conjugate, wherein the modification does not alter the essential functional property of the conjugate, namely, the substantially improved serum half-life, MRT and/or serum clearance rate as compared to that of the parental antibody fragment from which the conjugate is derived. In one embodiment, the invention provides any of the above-described conjugates modified to incorporate one or more nonproteinaceous functional group(s). For example, the conjugate can be modified to incorporate nonproteinaceous labels or reporter molecules, such as radiolabels, including any radioactive substance used in medical treatment or imaging or used as an effector function or tracer in an animal model, such as radioisotopic labels ^{99}Tc, ^{90}Y,^{111}In, ^{32}P, ^{14}C, ^{125}I, ^{3}H, ^{131}I, ^{11}C, ^{15}O, ^{13}N, ^{18}F, ^{35}S, ^{51}Cr, ^{57}To, ^{226}Ra, ^{60}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{234}Th, ^{40}K, and the like, non-radioisotopic labels such as ^{157}Gd, ^{55}Mn, ^{52}Tr, ^{56}Fe, etc., fluroescent or chemiluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthaladehyde, fluorescamine, ^{152}Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridimium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels, stable free radicals, and the like.

[0162] Conventional methods are available to bind these labels covalently to the polypeptide antibody fragment or polymer component of the conjugate. In one aspect, any conjugate of the invention is modified by derivatizing the antibody fragment component with any of the above-described non-proteinaceous labels, wherein the label is directly or indirectly (through a coupling agent) attached to the antibody fragment, and wherein such derivatization of the antibody fragment does not contribute or introduce any polymer moiety into the molecular structure of the conjugate. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like can be used to tag the antibody fragment with the above-described fluorescent or chemiluminescent labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry), Morrison, Meth. Enzymol., 32b, 103 (1974), Svyanen et al., J. Biol. Chem., 284, 3762 (1973), and Bolton and Hunter, Biochem. J., 133, 529 (1973).

[0163] In the case of embodiments utilizing radiolabels, both direct and indirect labeling can be used to incorporate the selected radionuclide into the conjugate. As used herein in the context of radiolabeling, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to the antibody fragment moiety or polymer moiety of the conjugate and at least one raidonuclide is inserted into the chelating agent. Preferred chelating agents and radionuclides are set forth in Srivagtava, S.C. and Mease, R.C., "Progress in Research on Ligands, Nuclides and Techniques for Labeling Monoclonal Antibodies," Nucl. Med. Bio., 18(6): 589-603 (1991). A particularly preferred chelating agent is 1-isothiocycmatobenzyl-3-methyldiothelene triaminepent acetic acid ("MX-DTPA"). As used herein in the context of radiolabeling, the phrases "direct labeling" and "direct labeling approach" both mean that a radionuclide is covalently attached directly to the antibody fragment moiety (typically via an amino acid residue)

or to the polymer moiety of the conjugate. Preferred radionuclides for use in direct labeling of conjugate are provided in Srivagtava and Mease, supra. In one embodiment, the conjugate is directly labeled with ^{131}I covalently attached to tyrosine residues. In another embodiment, the antibody fragment component of the conjugate is directly or indirectly labeled with any of the above-described radiolabels, wherein such labeling of the antibody fragment does not contribute or introduce any polymer moiety into the molecular structure of the conjugate.

d. Therapeutic Compositions and Administration of Large Effective Size Conjugates

[0164] The conjugate of the invention is useful for treating the disease indications that are treated with the parent intact antibody. For example, a conjugate derived from an anti-IL-8 antibody or fragment is useful in the treatment of inflammatory disorders as described in Section (II)(5)(B) below. Therapeutic formulations of the conjugate of the invention can be prepared by utilizing the same procedures described for the formulation of the anti-IL-8 antibodies and fragments of the invention in Section (II)(5)(B) below. The conjugate of the invention can be administered in place of the parent antibody for a given disease indication by modifying the formulation, dosage, administration protocol, and other aspects of a therapeutic regimen as required by the different pharmacodynamic characteristics of the conjugate and as dictated by common medical knowledge and practice.

e. Reagent Uses for Large Effective Size Conjugates

[0165] The conjugate of the invention also finds application as a reagent in an animal model system for in vivo study of the biological functions of the antigen recognized by the conjugate. The conjugate would enable the practitioner to inactivate or detect the cognate antigen in circulation or in tissue for a far greater period of time than would be possible with art-known constructs while removing any Fc interaction (which could attend the use of an intact antibody) from the system. In addition, the increased half-life of the conjugate of the invention can be applied advantageously to the induction of tolerance for the underivatized antibody fragment in a test animal by employing the Wie et al., Int. Archs. Allergy Appl. Immunol., 64: 84-99 (1981) method for allergen tolerization, which would permit the practitioner to repeatedly challenge the tolerized animal with the underivatized parental antibody fragment without generating an immune response against the parental fragment.

3. VARIANTS OF HUMANIZED MONOCLONAL ANTIBODIES AND ANTIBODY FRAGMENTS

[0166] An antibody fragment may comprise a heavy chain that is fused to or contains a leucine zipper sequence. The leucine zipper can increase the affinity or production efficiency of the antibody or antibody fragment of interest. Suitable leucine zipper sequences include the jun and fos leucine zippers taught by Kostelney *el al.*, J. Immunol., 148: 1547-1553 (1992) and the GCN4 leucine zipper described in the Examples below.

Bispecific Antibodies

[0167] Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities may be for IL-8, the other one for any other antigen. For example, bispecific antibodies specifically binding a IL-8 and neurotrophic factor, or two different types of IL-8 polypeptides are within the scope of the present invention.

[0168] Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature **305**:537 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829 published 13 May 1993, and in Traunecker *et al.*, EMBO J. **10**: 3655 (1991).

[0169] According to a different and more preferred approach, antibody-variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant-domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1), containing the site necessary for light-chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the

maximum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the production of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. This asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. For further details of generating bispecific antibodies, see, for example, Suresh *et al.*, Methods in Enzymology **121**:210(1986).

[0170] According to another approach, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0171] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/00373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

[0172] Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science,* **229**: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

[0173] Recent progress has facilitated the direct recovery of Fab'-SH fragments from E. *coli,* which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.*, **175**: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

[0174] Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.*, **148**(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad Sci. USA*, **90**:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the VH and VL domains of one fragment are forced to pair with the complementary VL and VH domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.*, **152**:5368 (1994).

[0175] Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* **147**: 60 (1991).

4. Production of Humanized Monoclonal Antibody, Antibody Fragments, and Variants

[0176] The antibody fragments of the invention can be produced using any convenient antibody manufacturing process known in the art. Typical, are antibody or antibody fragment is made using recombinant expression systems. A multiple polypeptide chain antibody or antibody fragment species can be made in a single host cell expression system wherein the host cell produces each chain of the antibody or antibody fragment and assembles the polypeptide chains

into a multimeric structure to form the antibody or antibody fragment in vivo, followed by recovery of the antibody or antibody fragment from the host cell. For example, suitable recombinant expression systems for the production of complete antibody or antibody fragment are described in Lucas *et al.*, Nucleic Acids Res., 24: 1774-1779 (1996). Alternatively, the separate polypeptide chains of the desired antibody or antibody fragment can be made in separate expression host cells, separately recovered from the respective host cells, and then mixed in vitro under conditions permitting the formation of the multi-subunit antibody or antibody fragment of interest. For example, U.S. Pat. No. 4,816,567 to Cabilly *et al.* and Carter *et al.*, Bio/Technolog, 10: 163-167 (1992) provide methods for recombinant production of antibody heavy and light chains in separate expression hosts followed by assembly of antibody from separate heavy and light chains in vitro.

[0177] The following discussion of recombinant expression methods applies equally to the production of single chain antibody polypeptide species and multi-subunit antibody and antibody fragment species. All recombinant procedures for the production of antibody or antibody fragment provided below shall be understood to describe: (1) manufacture of single chain antibody species as the desired end-product; (2) manufacture of multi-subunit antibody or antibody fragment species by production of all subunits in a single host cell, subunit assembly in the host cell, optionally followed by host cell secretion of the multi-subunit end-product into the culture medium, and recovery of the multi-subunit end-product from the host cell and/or culture medium; and (3) manufacture of multi-subunit antibody or antibody fragment by production of subunits in separate host cells (optionally followed by host cell secretion of subunits into the culture medium), recovery of subunits from the respective host cells and/or culture media, followed by in vitro subunit assembly to form the multi-subunit end-product. In the case of a multi-subunit antibody or antibody fragment produced in a single host cell, it will be appreciated that production of the various subunits can be effected by expression of multiple polypeptide-encoding nucleic acid sequences carried on a single vector or by expression of polypeptide-encoding nucleic acid sequences carried on multiple vectors contained in the host cell.

A. Construction of DNA Encoding Humanized 6G4.2.5 Monoclonal Antibodies, Antibody Fragments, and Variants

[0178] Following the selection of the humanized antibody or antibody fragment of the invention according to the methods described above, the practitioner can use the genetic code to design DNAs encoding the desired antibody or antibody fragment. In one embodiment, codons preferred by the expression host cell are used in the design of a DNA encoding the antibody or antibody fragment of interest. DNA encoding the desired antibody or antibody fragment can be prepared by a variety of methods known in the art. These methods include, but are not limited to, chemical synthesis by any of the methods described in Engels *et al.*, Agnew. Chem. Int. Ed. Engl., 28: 716-734 (1989), the entire disclosure of which is incorporated herein by reference, such as the triester, phosphite, phosphoramidite and H-phosphonate methods. A variation on the above procedures contemplates the use of gene fusions, wherein the gene(s) encoding the antibody or antibody fragment is associated, in the vector, with a gene encoding another protein or a fragment of another protein. This results in the antibody or antibody fragment being produced by the host cell as a fusion with another protein. The "other" protein is often a protein or peptide which can be secreted by the cell, making it possible to isolate and purify the desired protein from the culture medium and eliminating the necessity of destroying the host cells which arises when the desired protein remains inside the cell. Alternatively, the fusion protein can be expressed intracellularly. It is advantageous to use fusion proteins that are highly expressed.

[0179] The use of gene fusions, though not essential, can facilitate the expression of heterologous proteins in *E. coli* as well as the subsequent purification of those gene products (Harris, T. J. R. in *Genetic Engineering,* Williamson, R., Ed., Academic, London, Vol. 4, p. 127(1983); Uhlen, M. & Moks, T., *Methods Enzymol.* **185**:129-143 (1990)). Protein A fusions are often used because the binding of protein A, or more specifically the Z domain of protein A, to IgG provides an "affinity handle" for the purification of the fused protein (Nilsson, B. & Abrahmsen, L. *Methods Enzymol.* **185**:144-161 (1990)). It has also been shown that many heterologous proteins are degraded when expressed directly in *E. coli,* but are stable when expressed as fusion proteins (Marston, F. A. O., *Biochem J.* **240**: 1 (1986)).

[0180] Fusion proteins can be cleaved using chemicals, such as cyanogen bromide, which cleaves at a methionine, or hydroxylamine, which cleaves between an Asn and Gly. Using standard recombinant DNA methodology, the nucleotide base pairs encoding these amino acids may be inserted just prior to the 5' end of the antibody or antibody fragment gene(s).

[0181] Alternatively, one can employ proteolytic cleavage of fusion proteins, which has been recently reviewed (Carter, P. (1990) in *Protein Purification: From Molecular Mechanisms to Large-Scale Processes,* Ladisch, M. R., Willson, R. C., Painton, C. C., and Builder, S. E., eds., American Chemical Society Symposium Series No. 427, Ch 13, 181-193).

[0182] Proteases such Factor Xa, thrombin, subtilisin and mutants thereof, have been successfully used to cleave fusion proteins. Typically, a peptide linker that is amenable to cleavage by the protease used is inserted between the "other" protein (e.g., the Z domain of protein A) and the protein of interest, such as humanized anti-IL-8 antibody or antibody fragment. Using recombinant DNA methodology, the nucleotide base pairs encoding the linker are inserted between the genes or gene fragments coding for the other proteins. Proteolytic cleavage of the partially purified fusion

protein containing the correct linker can then be carried out on either the native fusion protein, or the reduced or denatured fusion protein.

[0183] Various techniques are also available which may now be employed to produce variant humanized antibodies or antibody fragments, which encodes for additions, deletions, or changes in amino acid sequence of the resultant protein(s) relative to the parent humanized antibody or antibody fragment.

[0184] By way of illustration, with expression vectors encoding humanized antibody or antibody fragment in hand, site specific mutagenesis (Kunkel *et al.*, *Methods Enzymol.* **204**:125-139 (1991); Carter, P., *et al.*, *Nucl. Acids. Res.* **13**:4331 (1986); Zoller, M. J. *et al.*,*Nucl. Acids Res.* **10**:6487 (1982)), cassette mutagenesis (Wells, J. A., *et al.*, *Gene* **34**:315 (1985)), restriction selection mutagenesis (Wells, J. A., *et al.*, *Philos. Trans. R. Soc. London SerA* **317**, 415 (1986)) or other known techniques may be performed on the antibody or antibody fragment DNA. The variant DNA can then be used in place of the parent DNA by insertion into the aforementioned expression vectors. Growth of host bacteria containing the expression vectors with the mutant DNA allows the production of variant humanized antibodies or antibody fragments, which can be isolated as described herein.

B. Insertion of DNA into a Cloning Vehicle

[0185] The DNA encoding the antibody or antibody fragment is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on (1) whether it is to be used for DNA amplification or for DNA expression, (2) the size of the DNA to be inserted into the vector, and (3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

[0186] In general, a signal sequence may be a component of the vector, or it may be a part of the antibody or antibody fragment DNA that is inserted into the vector. Preferably, a heterologous signal sequence selected and fused to the antibody or antibody fragment DNA such that the signal sequence in the corresponding fusion protein is recognized, transported and processed (*i.e.*, cleaved by a signal peptidase) in the host cell's protein secretion system. In the case of prokaryotic host cells, the signal sequence is selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. In a preferred embodiment, the STII signal sequence is used as described in the Examples below. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gO signal, are available.

(ii) Origin of Replication Component

[0187] Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

[0188] Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

[0189] DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is homologous to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the antibody or antibody fragment DNA.

(iii) Selection Gene Component

[0190] Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli.*

[0191] One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, J. Molec. Appl. Genet., 1: 327 (1982)), mycophenolic acid (Mulligan *et al.*, Science, 209: 1422 (1980)) or hygromycin (Sugden *et al.*, Mol. Cell. Biol., 5: 410-413 (1985)). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug (G418 or neomycin (geneticin), xgpt (mycophenolic acid), and hygromycin, respectively.)

[0192] Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody or antibody fragment nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the antibody or antibody fragment. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the antibody or antibody fragment are synthesized from the amplified DNA.

[0193] For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the antibody or antibody fragment. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the antibody or antibody fragment, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

[0194] A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7. Stinchcomb *et al.*, Nature, 282: 39 (1979); Kingsman *et al.*, Gene, 7: 141 (1979); or Tschemper *et al.*, Gene, 10: 157 (1980). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, Genetics, 85: 12 (1977). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

(iv) Promoter Component

[0195] Expression vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody or antibody fragment nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the antibody or antibody fragment encoding sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known.

[0196] Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.*, Nature, 275: 615 (1978); and Goeddel *et al.*, Nature, 281: 544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 (1980) and EP 36,776) and hybrid promoters such as

the tac promoter (deBoer *et al.*, Proc. Natl. Acad. Sci. USA, 80: 21-25 (1983)). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker to operably ligate them to DNA encoding the antibody or antibody fragment (Siebenlist *et al.*, Cell, 20: 269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgamo (S.D.) sequence operably linked to the DNA encoding the antibody or antibody fragment.

[0197] Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, J. Biol. Chem., 255: 2073 (1980)) or other glycolytic enzymes (Hess *et al.*, J. Adv. Enzyme Reg., 7: 149 (1968); and Holland, Biochemistry, 17: 4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

[0198] Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

[0199] Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

[0200] Vector driven transcription of antibody or antibody fragment encoding DNA in mammalian host cells can be controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

[0201] The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, 273: 113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells, Reyes *et al.*, Nature, 297: 598-601 (1982) on expression of human -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon 1 gene in cultured mouse and rabbit cells, and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

[0202] Transcription of a DNA encoding antibody or antibody fragment by higher eukaryotic host cells is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins *et al.*, Proc. Natl. Acad. Sci. USA, 78: 993 (1981)) and 3' (Lusky *et al.*, Mol. Cell Bio., 3: 1108 (1983)) to the transcription unit, within an intron (Banerji *et al.*, Cell, 33: 729 (1983)) as well as within the coding sequence itself (Osborne *et al.*, Mol. Cell Bio., 4: 1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody or antibody fragment DNA, hut is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

[0203] Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) can also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the antibody or antibody fragment. The 3' untranslated regions also include transcription termination sites.

[0204] Suitable vectors containing one or more of the above listed components and the desired coding and control sequences are constructed by standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

[0205] For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res., 9: 309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology, 65: 499 (1980).

[0206] Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the antibody or antibody fragment. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector.

[0207] Other methods, vectors, and host cells suitable for adaptation to the synthesis of the antibody or antibody fragment in recombinant vertebrate cell culture are described in Gething *et al.*, Nature, 293: 620-625 (1981); Mantei *et al.*, Nature, 281: 40-46 (1979); Levinson *et al.*, EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the IgE peptide antagonist is pRK5 (EP pub. no. 307,247) or pSV16B (PCT pub. no. WO 91/08291 published 13 June 1991).

C. Selection and Transformation of Host Cells

[0208] Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli, Bacilli* such as *B. subtilis, Pseudomonas* species such as *P. aeruginosa, Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* 1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. In a preferred embodiment, the E. coli strain 49D6 is used as the expression host as described in the Examples below. Review articles describing the recombinant production of antibodies in bacterial host cells include Skerra *et al.*, Curr. Opinion in Immunol., 5: 256 (1993) and Pluckthun, Immunol. Revs., 130: 151 (1992).

[0209] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors containing antibody or antibody fragment DNA. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *S. pombe* (Beach and Nurse, Nature, 290: 140 (1981)), *Kluyveromyces lactis* (Louvencourt *et al.*, J. Bacteriol., 737 (1983)), *yarrowia* (EP 402,226), *Pichia pastoris* (EP 183,070), *Trichoderma reesia* (EP 244,234), *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76: 5259-5263 (1979)), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112: 284-289 (1983); Tilburn *et al.*, Gene, 26: 205-221 (1983); Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475-479 (1985)).

[0210] Host cells derived from multicellular organisms can also be used in the recombinant production of antibody or antibody fragment. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow *et al.*, Bio/Technology, 6: 47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow, J.K. *et al.*, 8: 277-279 (Plenum Publishing, 1986), and Maeda *et al.*, Nature, 315: 592-594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spudoptera frugiperda* cells.

[0211] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts.

Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the antibody or antibody fragment DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding antibody or antibody fragment is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the antibody or antibody fragment DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA *780* gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published 21 June 1989.

[0212] Vertebrate cell culture is preferred for the recombinant production of full length antibodies. The propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36: 59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W 138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells. Myeloma cells that do not otherwise produce immunoglobulin protein are also useful host cells for the recombinant production of full length antibodies.

[0213] Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[0214] Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ precipitation and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

[0215] Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook *et al.*, *supra*, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact., 130: 946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA), 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or by protoplast fusion may also be used.

D. Culturing the Host Cells

[0216] Prokaryotic cells used to produce the antibody or antibody fragment are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

[0217] The mammalian host cells used to produce the antibody or antibody fragment can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz., 58: 44 (1979), Barnes and Sato, Anal. Biochem., 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temper-

ature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

[0218] The host cells referred to in this disclosure encompass cells in *in vitro* culture as well as cells that are within a host animal.

E. Detecting Gene Amplification/Expression

[0219] Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

[0220] Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu *et al.*, Am. J. Clin. Path., 75: 734-738 (1980).

F. Purification of the Antibody or Antibody Fragment

[0221] In the case of a host cell secretion system, the antibody or antibody fragment is recovered from the culture medium. Alternatively, the antibody can be produced intracellularly, or produced in the periplasmic space of a bacterial host cell. If the antibody is produced intracellularly, as a first step, the host cells are lysed, and the resulting particulate debris is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* **10**:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

[0222] The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma1$, $\gamma2$, or $\gamma4$ heavy chains (Lindmark *et al., J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma3$ (Guss *et al., EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin Sepharose™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

[0223] Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (e.g. from about 0-0.25M salt).

G. Production of Antibody Fragments

[0224] Various techniques have been developed for the production of the humanized antibody fragments of the

invention, including Fab, Fab', Fab'-SH, or $F(ab')_2$ fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.*, Morimoto *et al.*, *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter *et al.*, Bio/Technology, 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

5. Uses of Anti-IL-8 Antibodies

A. Diagnostic Uses

[0225] For diagnostic applications requiring the detection or quantitation of IL-8, the antibodies or antibody fragments of the invention typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety can be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I, ^{32}P, ^{14}C, or ^{3}H; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

[0226] Any method known in the art for separately conjugating the antibody or antibody fragment to the detectable moiety can be employed, including those methods described by Hunter *et al.*, Nature 144:945 (1962); David *et al.*, Biochemistry 13:1014 (1974); Pain *et al.*, J. Immunol. Meth. 40:219 (1981); and Nygren, J. Histochem. and Cytochem. 30:407 (1982).

[0227] The antibody fragments of the present invention can be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. For example, see Zola, Monoclonal Antibodies: A Manual of Techniques, pp. 147-158 (CRC Press, Inc., 1987).

[0228] Competitive binding assays rely on the ability of a labeled standard (which can be a IL-8 or an immunologically reactive portion thereof) to compete with the test sample analyte (IL-8) for binding with a limited amount of antibody or antibody fragment. The amount of IL-8 in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies or antibody fragments generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies can conveniently be separated from the standard and analyte which remain unbound.

[0229] Sandwich assays involve the use of two antibodies, each capable of binding to a different antigenic portion, or epitope, of the protein (IL-8) to be detected. In a sandwich assay, the test sample . analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex (U.S. Patent No. 4,376,110). The second antibody can itself be labeled with a detectable moiety (direct sandwich assays) or can be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme (e.g., horseradish peroxidase).

[0230] IL-8 antibody fragments also are useful for the affinity purification of IL-8 from recombinant cell culture or natural sources. For example, they can be fixed to a solid support by techniques well known in the art so as to purify IL-8 from a source such as culture supernatant or tissue.

B. Therapeutic Compositions and Administration of Anti-IL-8 Antibody

[0231] The humanized anti-IL-8 antibody fragments of the invention are useful in the treatment of inflammatory disorders, such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colitis, and rheumatoid arthritis.

[0232] Therapeutic formulations of the humanized anti-IL-8 antibody fragments are prepared for storage by mixing the antibody fragment having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, supra), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

[0233] The humanized anti-IL-8 antibody fragment to be used for in vivo administration must be sterile. This is readily

accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The humanized anti-IL-8 antibody fragment ordinarily will be stored in lyophilized form or in solution.

[0234] Therapeutic humanized anti-IL-8 antibody fragment compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

[0235] The route of humanized anti-IL-8 antibody fragment administration is in accord with known methods, e.g., inhalation, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, by enema or suppository, or by sustained release systems as noted below. Preferably it is given systemically or at a site of inflammation.

[0236] Suitable examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers 22:547 (1983)), poly (2-hydroxyethyl-methacrylate) (Langer *et al.*, J. Biomed. Mater. Res. 15:167 (1981) and Langer, Chem. Tech. 12:98 (1982)), ethylene vinyl acetate (Langer *et al.*, supra) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release humanized anti-IL-8 antibody fragment compositions also include liposomally entrapped antibody or antibody fragment. Liposomes containing an antibody fragment are prepared by methods known per se: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. U.S.A. 82:3688 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. U.S. A. 77:4030 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mole percent cholesterol, the selected proportion being adjusted for the most efficacious antibody or antibody fragment therapy.

[0237] An "effective amount" of the humanized anti-IL-8 antibody fragment to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the humanized anti-IL-8 antibody fragment until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

[0238] In the treatment and prevention of an inflammatory disorder with a humanized anti-IL-8 antibody fragment of the invention, the composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the antibody, the particular type of antibody, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the inflammatory disorder, including treating acute or chronic respiratory diseases and reducing inflammatory responses. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

[0239] As a general proposition, the initial pharmaceutically effective amount of the antibody fragment administered parenterally per dose will be in the range of about 0.1 to 50 mg/kg of patient body weight per day, with the typical initial range of antibody used being 0.3 to 20 mg/kg/day, more preferably 0.3 to 15 mg/kg/day.

[0240] As noted above, however, these suggested amounts of antibody fragment are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above.

[0241] The antibody fragment need not be, but is optionally formulated with one or more agents currently used to prevent or treat the inflammatory disorder in question. For example, in rheumatoid arthritis, it can be given in conjunction with a glucocorticosteroid. The effective amount of such other agents depends on the amount of antibody fragment present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

[0242] Some of the following examples illustrate the invention. Others provide useful background. They are offered by way of illustration and not by way of limitation.

EXAMPLES

A. GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN IL-8

[0243] Balb/c mice were immunized in each hind footpad or intraperitoneally with 10 μg of recombinant human IL-8 (produced as a fusion of (ser-IL-8)$_{72}$ with ubiquitin (Hebert *et al.* J. Immunology 145:3033-3040 (1990)); IL-8 is available commercially from PeproTech, Inc., Rocky Hill, NJ) resuspended in MPL/TDM (Ribi Immunochem. Research Inc.,

Hamilton, MT) and boosted twice with the same amount of IL-8. In these experiments, "IL-8" is intended to mean (ser-IL-8)$_{72}$ unless otherwise specified. A final boost of 10 μg of IL-8 was given 3 days before the fusion. Spleen cells or popliteal lymph node cells were fused with mouse myeloma P3X63Ag8U.1 (ATCC CRL1597), a non-secreting clone of the myeloma P3X63Ag8, using 35% polyethylene glycol as described before. Ten days after the fusion, culture supernatant was screened for the presence of monoclonal antibodies to IL-8 by ELISA.

[0244] The ELISA was performed as follows. Nunc 96-well immunoplates (Flow Lab, McLean, VA) were coated with 50 μl/well of 2 μg/ml IL-8 in phosphate-buffered saline (PBS) overnight at 4°C. The remaining steps were carried out at room temperature. Nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA) for 1 hour (hr). Plates were then incubated with 50 μl/well of hybridoma culture supernatants from 672 growing parental fusion wells for 1 hr, followed by the incubation with 50 μl/well of 1:1000 dilution of a 1 mg/ml stock solution of alkaline phosphatase-conjugated goat anti-mouse 1g (Tago Co., Foster City, CA) for I hr. The level of enzyme-linked antibody bound to the plate was determined by the addition of 100 μl/well of 0.5 mg/ml of r-nitrophenyl phosphate in sodium bicarbonate buffer, pH 9.6. The color reaction was measured at 405 nm with an ELISA plate reader (Titertrek Multiscan, Flow Lab, McLean, VA). Between each step, plates were washed three times in PBS containing 0.05% Tween 20.

[0245] As a general proposition, the initial pharmaceutically effective amount of the antibody or antibody fragment administered parenterally per dose will be in the range of about 0.1 to 50 mg/kg of patient body weight per day, with the typical initial range of antibody used being 0.3 to 20 mg/kg/day, more preferably 0.3 to 15 mg/kg/day.

[0246] As noted above, however, these suggested amounts of antibody or antibody fragment are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above.

[0247] The antibody or antibody fragment need not be, but is optionally formulated with one or more agents currency used to prevent or treat the inflammatory disorder in question. For example, in rheumatoid arthritis, the antibody can be given in conjunction with a glucocorticosteroid. The effective amount of such other agents depends on the amount of antibody or antibody fragment present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

[0248] The following examples are offered by way of illustration and not by way of limitation. The disclosures of all references cited in the specification, and the disclosures of all citations in such references, are expressly incorporated herein by reference.

EXAMPLES

A. GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN IL-8

[0249] Balb/c mice were immunized in each hind footpad or intraperitoneally with 10 μg of recombinant human IL-8 (produced as a fusion of (ser-IL-8)$_{72}$ with ubiquitin (Hebert *et al.* J. Immunology 145:3033-3040 (1990)); IL-8 is available commercially from PeproTech, Inc., Rocky Hill, NJ) resuspended in MPL/TDM (Ribi Immunochem. Research Inc., Hamilton, MT) and boosted twice with the same amount of IL-8. In these experiments, "IL-8" is intended to mean (ser-IL-8)$_{72}$ unless otherwise specified. A final boost of 10 μg of IL-8 was given 3 days before the fusion. Spleen cells or popliteal lymph node cells were fused with mouse myeloma P3X63Ag8U.1 (ATCC CRL1597), a non-secreting clone of the myeloma P3X63Ag8, using 35% polyethylene glycol as described before. Ten days after the fusion, culture supernatant was screened for the presence of monoclonal antibodies to IL-8 by ELISA.

[0250] The ELISA was performed as follows. Nunc 96-well immunoplates (Flow Lab, McLean, VA) were coated with 50 μl/well of 2 μg/ml IL-8 in phosphate-buffered saline (PBS) overnight at 4°C. The remaining steps were carried out at room temperature. Nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA) for 1 hour (hr). Plates were then incubated with 50 μl/well of hybridoma culture supernatants from 672 growing parental fusion wells for I hr, followed by the incubation with 50 μl/well of 1:1000 dilution of a 1 mg/ml stock solution of alkaline phosphatase-conjugated goat anti-mouse 1g (Tago Co., Foster City, CA) for I hr. The level of enzyme-linked antibody bound to the plate was determined by the addition of 100 μl/well of 0.5 mg/ml of r-nitrophenyl phosphate in sodium bicarbonate buffer, pH 9.6. The color reaction was measured at 405 nm with an ELISA plate reader (Titertrek Multiscan, Flow Lab, McLean, VA). Between each step, plates were washed three times in PBS containing 0.05% Tween 20.

[0251] Culture supernatants which promoted 4-fold more binding of IL-8 than did control medium were selected as positives. According to this criterion, 16 of 672 growing parental fusion wells (2%) were positive. These positive hybridoma cell lines were cloned at least twice by using the limiting dilution technique.

[0252] Seven of the positive hybridomas were further characterized as follows. The isotypes of the monoclonal antibodies were determined by coating Nunc 96-well immunoplates (Flow Lab, McLean, VA) with IL-8 overnight, blocking with BSA, incubating with culture supernatants followed by the addition of predetermined amount of isotype-specific alkaline phosphatase-conjugated goat anti-mouse 1g (Fisher Biotech, Pittsburgh, PA). The level of conjugated anti-

bodies bound to the plate was determined by the addition of r-nitrophenyl phosphate as described above.

[0253] All the monoclonal antibodies tested belonged to either IgG_1 or IgG_2 immunoglobulin isotype. Ascites fluid containing these monoclonal antibodies had antibody titers in the range of 10,000 to 100,000 as determined by the reciprocal of the dilution factor which gave 50% of the maximum binding in the ELISA.

[0254] To assess whether these monoclonal antibodies bound to the same epitopes, a competitive binding ELISA was performed. At a ratio of biotinylated mAb to unlabeled mAb of 1:100, the binding of biotinylated mAb 5.12.14 was significantly inhibited by its homologous mAb but not by mAb 4.1.3, while the binding of biotinylated mAb 4.1.3 was inhibited by mAb 4.1.3 but not by mAb 5.12.14. Monoclonal antibody 5.2.3 behaved similarly to mAb 4.1.3, while monoclonal antibodies 4.8 and 12.3.9 were similar to mAb 5.12.14. Thus, mAb 4.1.3 and mAb 5.2.3 bind to a different epitope(s) than the epitope recognized by monoclonal antibodies 12.3.9, 4.8 and 5.12.14.

[0255] Immunodot blot analysis was performed to assess antibody reactivity to IL-8 immobilized on nitrocellulose paper. All seven antibodies recognized IL-8 immobilized on paper, whereas a control mouse IgG antibody did not.

[0256] The ability of these monoclonal antibodies to capture soluble ^{125}I-IL-8 was assessed by a radioimmune precipitation test (RIP). Briefly, tracer ^{125}I-IL-8 (4×10^4 cpm) was incubated with various dilutions of the monoclonal anti-IL-8 antibodies in 0.2 ml of PBS containing 0.5% BSA and 0.05% Tween 20 (assay buffer) for I hr at room temperature. One hundred microliters of a predetermined concentration of goat anti-mouse 1g antisera (Pel-Freez, Rogers, AR) were added and the mixture was incubated at room temperature for 1 hr. Immune complexes were precipitated by the addition of 0.5 ml of 6% polyethylene glycol (M.W. 8000) kept at 4°C. After centrifugation at 2,000 x g for 20 min at 4°C, the supernatant was removed by aspiration and the radioactivity remaining in the pellet was counted in a gamma counter. Percent specific binding was calculated as (precipitated cpm - background cpm)/ (total cpm - background cpm). Monoclonal antibodies 4.1.3, 5.2.3, 4.8, 5.12.14 and 12.3.9 captured ^{125}I-IL-8 very efficiently, while antibodies 9.2.4 and 8.9.1 were not able to capture soluble ^{125}I-IL-8 in the RIP even though they could bind to IL-8 coated onto ELISA plates (Table 1).

[0257] The dissociation constants of these monoclonal antibodies were determined using a competitive binding RIP assay. Briefly, competitive inhibition of the binding each antibody to ^{125}I-IL-8 (20,000-40,000 cpm per assay) by various amounts of unlabeled IL-8 was determined by the RIP described above. The dissociation constant (affinity) of each mAb was determined by using Scatchard plot analysis (Munson, *et al.*, Anal. Biochem. 107:220 (1980)) as provided in the VersaTerm-PRO computer program (Synergy Software, Reading, PA).The K_d's of these monoclonal antibodies (with the exception of 9.2.4. and 8.9.1) were in the range from 2×10^{-8} to 3×10^{-10} M. Monoclonal antibody 5.12.14 with a K_d of 3×10^{-10} M showed the highest affinity among all the monoclonal antibodies tested (Table 3).

Table 3.

Characterization of Anti-tL-8 Monoclonal Antibodies				
Antibody	%Specific Binding to IL-8	K_d (M)	Isotype	pI
4.1.3	58	2×10^{-9}	IgG_1	4.3-6.1
5.2.3	34	2×10^{-8}	IgG_1	5.2-5.6
9.2.4	1	-	IgG_1	7.0-7.5
8.9.1	2	-	IgG_1	6.8-7.6
4.8	62	3×10^{-8}	IgG_{2a}	6.1-7.1
5.12.14	98	3×10^{-10}	IgG_{2a}	6.2-7.4
12.3.9	86	2×10^{-9}	IgG_{2a}	6.5-7.1

[0258] To assess the ability of these monoclonal antibodies to neutralize IL-8 activity, the amount of ^{125}I-IL-8 bound to human neutrophils in the presence of various amounts of culture supernatants and purified monoclonal antibodies was measured. Neutrophils were prepared by using Mono-Poly Resolving Medium (M-PRM) (Flow Lab. Inc., McLean, VA). Briefly fresh, heparinized human blood was loaded onto M-PRM at a ratio of blood to medium, 3.5:3.0, and centrifuged at 300 x g for 30 min at room temperature. Neutrophils enriched at the middle layer were collected and washed once in PBS. Such a preparation routinely contained greater than 95% neutrophils according to the Wright's Giemsa staining. The receptor binding assay was done as follows. 50 µl of ^{125}I-IL-8 (5 ng/ml) was incubated with 50 µl of unlabeled IL-8 (100 µg/ml) or monoclonal antibodies in PBS containing 0.1% BSA for 30 min at room temperature. The mixture was then incubated with 100 µl of neutrophils (10^7 cells/ml) for 15 min at 37°C. The ^{125}I-IL-8 bound was separated from the unbound material by loading mixtures onto 0.4 ml of PBS containing 20% sucrose and 0.1% BSA and by centrifugation at 300 x g for 15 min. The supernatant was removed by aspiration and the radioactivity associated with the pellet was counted in a gamma counter.

[0259] Monoclonal antibodies 4.1.3, 5.2.3, 4.8, 5.12.14, and 12.3.9 inhibited greater than 85% of the binding of IL-8 to human neutrophils at a 1:25 molar ratio of IL-8 to mAb. On the other hand, monoclonal antibodies 9.2.4 and 8.9.1 appeared to enhance the binding of IL-8 to its receptors on human neutrophils. Since a control mouse IgG also enhanced the binding of IL-8 on neutrophils, the enhancement of IL-8 binding to its receptors by mAb 9.2.4 and 8.9.1 appears to be nonspecific. Thus, monoclonal antibodies, 4.1.3, 5.1.3, 4.8, 5.12.14, and 12.3.9 are potential neutralizing monoclonal antibodies while monoclonal antibodies 8.9.1 and 9.2.4 are non-neutralizing monoclonal antibodies.

[0260] The ability of the anti-IL-8 antibodies to block neutrophil chemotaxis induced by IL-8 was tested as follows. Neutrophil chemotaxis induced by IL-8 was determined using a Boyden chamber method (Larsen, *et al.* Science 243: 1464 (1989)). One hundred μl of human neutrophils (10^6 cells/ml) resuspended in RPMI containing 0.1% BSA were placed in the upper chamber and 29 μl of the IL-8 (20 nM) with or without monoclonal antibodies were placed in the lower chamber. Cells were incubated for 1 hr at 37°C. Neutrophils migrated into the lower chamber were stained with Wright's Giemsa stain and counted under the microscope (100x magnification). Approximately 10 different fields per experimental group were examined. Neutralizing monoclonal antibodies 5.12.14 and 4.1.3 blocked almost 70% of the neutrophil chemotactic activity of IL-8 at 1:10 ratio of IL-8 to mAb.

[0261] The isoelectric focusing (IEF) pattern of each mAb was determined by applying purified antibodies on an IEF polyacrylamide gel (pH 3-9, Pharmacia) using the Fast gel system (Pharmacia, Piscataway, NJ). The IEF gel was pretreated with pharmalyte containing 1% Triton X100 (Sigma, St. Louis, MO) for 10 min before loading the samples. The IEF pattern was visualized by silver staining according to the instructions from the manufacturer. All of the monoclonal antibodies had different IEF patterns, confirming that they originated from different clones. The pl values for the antibodies are listed in Table 3.

[0262] All these monoclonal antibodies bound equally well to both (ala-IL-8)77 and (ser-IL-8)72 forms of IL-8. Because IL-8 has greater than 30% sequence homology with certain other members of the platelet factor 4 (PF4) family of inflammatory cytokines such as β-TG (Van Damme *et al.*, Eur. J. Biochem. 181:337(1989); Tanaka *et al.*, FEB 236 (2):467 (1988)) and PF4 (Deuel *et al.*, Proc. Natl. Acad. Sci. U.S.A. 74:2256 (1977)), they were tested for possible cross reactivity to β-TG and PF4, as well as to another neutrophil activating factor, C5a. No detectable binding to any of these proteins was observed, with the exception of mAb 4.1.3, which had a slight cross reactivity to β-TG.

[0263] One of the antibodies, mAb 5.12.14, was further studied to determine whether it could block the IL-8 mediated release of elastase by neutrophils. Briefly, human neutrophils were resuspended in Hanks balanced salt solution (Gibco, Grand Island, NY) containing 1.0% BSA, Fraction V (Sigma, St. Louis, MO), 2 mg/ml alpha-D-glucose (Sigma), 4.2 mM sodium bicarbonate (Sigma) and 0.01 M HEPES, pH 7.1 (JRH Bioscience, Lenexa, KS). A stock of cytochalasin B (Sigma) was prepared (5 mg/ml in dimethylsulfoxide (Sigma) and stored at 2-8°C. Cytochalasin B was added to the neutrophil preparation to produce a final concentration of 5 μg/ml, and incubated for 15 min at 37°C. Human IL-8 was incubated with mAb 5.12.14 (20 μl), or a negative control antibody, in 1 ml polypropylene tubes (DBM Scientific, San Femando, CA) for 30 min at 37°C. The final assay concentrations of IL-8 were 50 and 500 nM. The monoclonal antibodies were diluted to produce the following ratios (IL-8:Mab): 1:50, 1:10, 1:2, 1:1, and 1:0.25. Cytochalasin B-treated neutrophils were added (100 μl/tube) and incubated for 2 hours at 25°C. The tubes were centrifuged (210 X g, 2-8°C) for 10 min, and supernatants were transferred to 96 well tissue culture plates (30 μl/well). Elastase substrate stock, 10 mM methoxysuccinyl-alanyl-alanyl-propyl-valyl-p-nitroanilide (Calbiochem, La Jolla, CA) in DMSO was prepared and stored at 2-8°C. Elastase substrate solution (1.2 mM substrate, 1.2 M NaCl (Mallinckrodt, Paris, Kentucky), 0.12 M HEPES pH 7.2 in distilled water) was added (170 μl/well) to the supernatants and incubated for 0.5 to 2 hours at 37°C (until control O.D. of 1.0 was reached). Absorbance was measured at 405 nm (SLT 340 ATTC plate reader, SLT Lab Instruments, Austria).

[0264] The results are shown in Figure 1. At a 1:1 ratio of IL-8 to mAb 5.12.14, the antibody was able to effectively block the release of elastase from neutrophils.

[0265] The hybridoma producing antibody 5.12.14 was deposited on February 15, 1993 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. HB 11553.

B. GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RABBIT IL-8

[0266] Antibodies against rabbit IL-8 were generated in essentially the same process as anti-human IL-8 antibodies using rabbit IL-8 as immunogen (kindly provided by C. Broaddus; see also Yoshimura *et al.* J. Immunol. 146:3483 (1991)). The antibody was characterized as described above for binding to other cytokines coated onto ELISA plates; no measurable binding was found to MGSA, fMLP, C5a, b-TG, TNF, PF4, or IL-1.

[0267] The hybridoma producing antibody 6G4.2.5 was deposited on September 28, 1994, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. HB 11722.

[0268] Recombinant human-murine chimeric Fabs for 5.12.14 and 6G4.2.5 were constructed as described below. A chimeric 6G.4.25 Fab is compared with a chimeric 5.12.14 Fab in detail below.

1. INHIBITION OF IL-8 BINDING TO HUMAN NEUTROPHILS BY 5.12.14-FAB AND 6G4 2.5-FAB

[0269] The ability of the two chimeric Fabs, 5.12.14-Fab and 6G4.2.5-Fab, to efficiently bind IL-8 and prevent IL-8 from binding to IL-8 receptors on human neutrophils was determined by performing a competition binding assay which allows the calculation of the IC_{50} - concentration required to achieve 50% inhibition of IL-8 binding.

[0270] Human neutrophils (5 X 10^5) were incubated for I hour at 4°C with 0.5nM ^{125}I-IL-8 in the presence of various concentrations (0 to 300 nM) of 5.12.14-Fab, 6G4.2.5-Fab, an isotype control (4D5-Fab) or unlabeled IL-8. After the incubation, the unbound ^{125}I-IL-8 was removed by centrifugation through a solution of 20% sucrose and 0.1% bovine serum albumin in phosphate buffered saline and the amount of ^{125}I-IL-8 bound to the cells was determined by counting the cell pellets in a gamma counter. Figure 2 demonstrates the inhibition of ^{125}I-IL-8 binding to neutrophils by unlabeled IL-8. Figure 3 demonstrates that a negative isotype matched Fab does not inhibit the binding of ^{125}I-IL-8 to human neutrophils. Both the anti-IL-8 Fabs, 5.12.14 Fab (Figure 4) and 6G.4.25 Fab (Figure 5) were able to inhibit the binding of ^{125}I-IL-8 to human neutrophils with an average IC_{50} of 1.6 nM and 7.5 nM, respectively.

2. INHIBITION OF IL-8-MEDIATED NEUTROPHIL CHEMOTAXIS BY 5.12.14-FAB AND 6G4.2.5-FA B

[0271] Human neutrophils were isolated, counted and resuspended at 5 x 10^6 cells/ml in Hank's balanced salt solution (abbreviated HBSS; without calcium and magnesium) with 0.1% bovine serum albumin. The neutrophils were labeled by adding calcein AM (Molecular Probe, Eugene, OR) at a final concentration of 2.0 µM. Following a 30 minute incubation at 37°C, cells were washed twice with HBSS-BSA and resuspended at 5 x 10^6 cells/ml.

[0272] Chemotaxis experiments were carried out in a Neuro Probe (Cabin John, MD) 96-well chamber, model MBB96. Experimental samples (buffer only control, IL-8 alone or IL-8 + Fabs) were loaded in a Polyfiltronics 96-well View plate (Neuro Probe Inc.) placed in the lower chamber. 100 µl of the calcein AM-labeled neutrophils were added to the upper chambers and allowed to migrate through a 5 micrometer porosity PVP free polycarbonate framed filter (Neuro Probe Inc.) toward the bottom chamber sample. The chemotaxis apparatus was then incubated for 40 to 60 minutes at 37°C with 5% CO_2. At the end of the incubation, neutrophils remaining in the upper chamber were aspirated and upper chambers were washed three times with PBS. Then the polycarbonate filter was removed, non-migrating cells were wiped off with a squeegee wetted with PBS, and the filter was air dried for 15 minutes.

[0273] The relative number of neutrophils migrating through the filter (Neutrophil migration index) was determined by measuring fluorescence intensity of the filter and the fluorescence intensity of the contents of the lower chamber and adding the two values together. Fluorescence intensity was measured with a CytoFluor 2300 fluorescent plate reader (Millipore Corp. Bedford, MA) configured to read a Coming 96-well plate using the 485-20 nm excitation filter and a 530-25 emission filter, with the sensitivity set at 3.

[0274] The results are shown in Figures 6 and 7. Figure 6 demonstrates the inhibition of human IL-8 mediated neutrophil chemotaxis by chimeric 6G4.2.5 and 5.12.14 Fabs. Figure 7 demonstrates the relative abilities of chimeric 6G4.2.5 and 5.12.14 Fabs to inhibit rabbit IL-8 mediated neutrophil chemotaxis.

3. INHIBITION OF IL-8-MEDIATED NEUTROPHIL ELASTASE RELEASE BY VARIOUS CONCENTRATIONS OF 6G4.2.5 AND 5.12.14 FABS

[0275] Blood was drawn from healthy male donors into heparinized syringes. Neutrophils were isolated by dextran sedimentation, centrifugation over Lymphocyte Separation Medium (Organon Teknika, Durham, NC), and hypotonic lysis of contaminating red blood cells as described by Berman *et al.* (J. Cell Biochem. 52:183 (1993)). The final neutrophil pellet was suspended at a concentration of 1 x 10^7 cells/ml in assay buffer, which consisted of Hanks Balanced Salt Solution (GIBCO, Grand Island, NY) supplemented with 1.0% BSA (fraction V, Sigma, St. Louis, MO), 2 mg/ml glucose, 4.2 mM sodium bicarbonate, and 0.01 M HEPES, pH 7.2. The neutrophils were stored at 4°C for not longer than 1 hr.

[0276] IL-8 (10 µl) was mixed with anti-IL-8 Fab, an isotype control Fab, or buffer (20 µl) in 1 ml polypropylene tubes and incubated in a 37°C water bath for 30 min. IL-8 was used at final concentrations ranging from 0.01 to 1000 nM in dose response studies (Figure 8) and at a final concentration of 100 nM in the experiments addressing the effects of the Fabs on elastase release (Figures 9 and 10). Fab concentrations ranged from approximately 20 nM to 300 nM, resulting in Fab:IL-8 molar ratios of 0.2: 1 to 3:1. Cytochalasin B (Sigma) was added to the neutrophil suspension at a concentration of 5 µg/ml (using a 5 mg/ml stock solution made up in DMSO), and the cells were incubated for 15 min in a 37°C water bath. Cytochalasin B-treated neutrophils (100 µl) were then added to the IL-8/Fab mixtures. After a 3 hr incubation at room temperature, the neutrophils were pelleted by centrifugation (200 x g for 5 min), and aliquots of the cell-free supernatants were transferred to 96 well plates (30 µl/well). The elastase substrate, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (Calbiochem, La Jolla, CA), was prepared as a 10 mM stock solution in DMSO and stored at 4°C. Elastase substrate working solution was prepared just prior to use (1.2 mM elastase substrate, 1.2

M NaCl, 0.12 M HEPES, pH 7.2), and 170 µl was added to each sample-containing well. The plates were placed in a 37°C tissue culture incubator for 30 min or until an optical density reading for the positive controls reached at least 1.0. Absorbance was measured at 405 nm using an SLT 340 plate reader (SLT Lab Instruments, Austria).

[0277] Figure 9 demonstrates the ability of the chimeric anti-IL-8 Fabs to inhibit elastase release from human neutrophils stimulated by human IL-8; Figure 10 demonstrates the relative abilities of the chimeric anti-IL-8 Fabs to inhibit elastase release from human neutrophils stimulated by rabbit IL-8.

C. MOLECULAR CLONING OF THE VARIABLE LIGHT AND HEAVY REGIONS OF THE MURINE 5.12.14 (ANTI-IL-9) MONOCLONAL ANTIBODY

[0278] Total RNA was isolated from 1 X 10^8 cells (hybridoma cell line ATCC HB-11722) using the procedure described by Chomczynski and Sacchi (Anal. Biochem. 162:156 (1987)). First strand cDNA was synthesized by specifically priming the mRNA with synthetic DNA oligonucleotides designed to hybridize with regions of the murine RNA encoding the constant region of the kappa light chain or the IgG2a heavy chain (the DNA sequence of these regions are published in Sequences of Proteins of Immunological Interest, Kabat, E. A. *et al.* (1991) NIH Publication 91-3242, V 1-3.). Three primers (SEQ ID NOS: 1-6) were designed for each of the light and heavy chains to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis (Figure 13). Amplification of the first strand cDNA to double-stranded (ds) DNA was accomplished using two sets of synthetic DNA oligonucleotide primers: one forward primer (SEQ ID NOS: 7-9) and one reverse primer (SEQ ID NO: 10) for the light chain variable region amplification (Figure 14) and one forward primer (SEQ ID NOS: 11-14) and one reverse primer (SEQ ID NOS: 11, 15, 14 and 13) for the heavy chain variable region amplification (Figure 15). The N-terminal sequence of the first eight amino acids of either the light or heavy chains of 5.12.14 was used to generate a putative murine DNA sequence corresponding to this region. (A total of 29 amino acids was sequenced from the N-terminus of both the light chain and heavy chain variable regions using the Edman degradation protein sequencing technique.) This information was used to design the forward amplification primers which were made degenerate in the third position for some codons to increase the chances of primer hybridization to the natural murine DNA codons and also included the unique restriction site, MluI, for both the light chain variable region forward primer and the heavy chain variable region forward primer to facilitate ligation to the 3' end of the STII element in the cloning vector. The reverse amplification primers were designed to anneal with the murine DNA sequence corresponding to a portion of the constant region of the light or heavy chains near the variable/constant junction. The light chain variable region reverse primer contained a unique BstBI restriction site and the heavy chain variable region reverse primer contained a unique ApaI restriction site for ligation to the 5' end of either the human IgG1 constant light or IgGI constant heavy regions in the vectors, pB 13.1 (light chain) and pB 14 (heavy chain). The polymerase chain reaction using these primer sets yielded DNA fragments of approximately 400 bp. The cDNA encoding the 5.12.14 light chain variable region was cloned into the vector pB13.1, to form pA51214VL and the 5.12.14 heavy chain variable region was cloned into the vector, pB14, to form pA51214VH. The cDNA inserts were characterized by DNA sequencing and are presented in the DNA sequence (SEQ ID NO: 16) and amino acid sequence (SEQ ID NO: 17) of Figure 16 (murine light chain variable region) and in the DNA sequence (SEQ ID NO: 18) and amino acid (SEQ ID NO: 19) of Figure 17 (murine heavy chain variable region).

D. CONSTRUCTION OF A 5.12.14 FAB VECTOR

[0279] In the initial construct, pA51214VL, the amino acids between the end of the 5.12.14 murine light chain variable sequence and the unique cloning site, BstBI, in the human IgG1 constant light sequence were of murine origin corresponding to the first 13 amino acids of the murine IgG1 constant region (Figure 16). Therefore, this plasmid contained a superfluous portion of the murine constant region separating the 5.12.14 murine light chain variable region and the human light chain IgG1 constant region. This intervening sequence would alter the amino acid sequence of the chimera and most likely produce an incorrectly folded Fab. This problem was addressed by immediately truncating the cDNA clone after A109 and re-positioning the BstBI site to the variable/constant junction by the polymerase chain reaction. Figure 18 shows the amplification primers used to make these modifications. The forward primer, VL.front (SEQ ID NO: 20), was designed to match the last five amino acids of the STII signal sequence, including the MluI cloning site, and the first 4 amino acids of the 5.12.14 murine light chain variable sequence. The sequence was altered from the original cDNA in the third position of the first two codons D1 (T to C) and 12 (C to T) to create a unique EcoRV cloning site which was used for later constructions. The reverse primer, VL.rear (SEQ ID NO: 21), was designed to match the first three amino acids of the human IgG1 constant light sequence and the last seven amino acids of the 5.12.14 light chain variable sequence which included a unique BstBI cloning site. In the process of adding the BstBI site, the nucleotide sequence encoding several amino acids were altered: L106 (TTG to CTT), K107 (AAA to CGA) resulting in a conservative amino acid substitution to arginine, and R108 (CGG to AGA). The PCR product encoding the modified 5.12.14 light chain variable sequence was then subcloned into pB13.1 in a two-part ligation. The MluI-BstBI digested

5.12.14 PCR product encoding the light chain variable region was ligated into MluI-BstBI digested vector to form the plasmid, pA51214VL'. The modified cDNA was characterized by DNA sequencing. The coding sequence for the 5.12.14 light chain is shown in Figure 19.

[0280] Likewise, the DNA sequence between the end of the heavy chain variable region and the unique cloning site, ApaI, in the human IgG1 heavy chain constant domain of pA51214VH was reconstructed to change the amino acids in this area from murine to human. This was done by the polymerase chain reaction. Amplification of the murine 5.12.14 heavy chain variable sequence was accomplished using the primers shown in Figure 18. The forward PCR primer (SEQ ID NO: 22) was designed to match nucleotides 867-887 in pA51214VH upstream of the ST11 signal sequence and the putative cDNA sequence encoding the heavy chain variable region and included the unique cloning site SpeI. The reverse PCR primer (SEQ ID NO: 23) was designed to match the last four amino acids of the 5.12.14 heavy chain variable sequence and the first six amino acids corresponding to the human IgGI heavy constant sequence which also included the unique cloning site, ApaI. The PCR product encoding the modified 5.12.14 heavy chain variable sequence was then subcloned to the expression plasmid, pMHM24.2.28 in a two-part ligation. The vector was digested with SpeI-ApaI and the SpeI-ApaI digested 5.12.14 PCR product encoding the heavy chain variable region was ligated into it to form the plasmid, pA51214VH'. The modified cDNA was characterized by DNA sequencing. The coding sequence for the 5.12.14 heavy chain is shown in the DNA sequence (SEQ ID NO: 26) and amino acid sequence (SEQ ID NO: 27) of Figures 20A-20B.

[0281] The first expression plasmid, pantiIL-8.1, encoding the chimeric Fab of 5.12.14 was made by digesting pA51214VH' with EcoRV and Bpu11021 to replace the EcoRV-Bpu11021 fragment with a EcoRV-Bpu11021 fragment encoding the murine 5.12.14 light chain variable region of pAS1214VL'. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 5.12.14.

[0282] Preliminary analysis of Fab expression using pantiIL-8.1 showed that the light and heavy chains were produced intracellularly but very little was being secreted into the periplasmic space of E. coli. To correct this problem, a second expression plasmid was constructed.

[0283] The second expression plasmid, pantiIL-8.2, was constructed using the plasmid, pmy187, as the vector. Plasmid pantiIL-8.2 was made by digesting pmy187 with MluI and SphI and the MluI (partial)-SphI fragment encoding the murine 5.12.14 murine-human chimeric Fab of pantiIL-8.1 was ligated into it. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 5.12.14.

[0284] The plasmid pantiIL-8.2 was deposited on February 10, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. ATCC 97056.

E. MOLECULAR CLONING OF THE VARIABLE LIGHT AND HEAVY REGIONS OF THE MURINE 6G4.2.5 MONOCLONAL ANTIBODY

[0285] Total RNA was isolated from 1×10^8 cells (hybridoma cell line 6G4.2.5) using the procedure described by Chomczynski and Sacchi (Anal. Biochem. 162:156 (1987)). First strand cDNA was synthesized by specifically priming the mRNA with synthetic DNA oligonucleotides designed to hybridize with regions of the murine RNA encoding the constant region of the kappa light chain or the IgG2a heavy chain (the DNA sequence of these regions are published in Sequences of Proteins of Immunological Interest, Kabat *et al.* (1991) NIH Publication 91-3242, V 1-3). Three primers (SEQ ID NOS: SEQ ID NOS: 1-6) were designed for each the light and heavy chains to increase the chances of primer hybridization and efficiency of first strand cDNA synthesis (Figure 21). Amplification of the first strand cDNA to double-stranded (ds) DNA was accomplished using two sets of synthetic DNA oligonucleotide primers: one forward primer (SEQ ID NOS: 28-30) and one reverse primer (SEQ ID NO: 31) for the light chain variable region amplification (Figure 22) and one forward primer (SEQ ID NOS: 32-33) and one reverse primer (SEQ ID NOS: 11,15,14 and 13) for the heavy chain variable region amplification (Figure 23). The N-terminal sequence of the first eight amino acids of either the light or heavy chains of 6G4.2.5 was used to generate a putative murine DNA sequence corresponding to this region. (A total of 29 amino acids were sequenced from the N-terminus of both the light chain and heavy chain variable regions using the Edman degradation protein sequencing technique.) This information was used to design the forward amplification primers which were made degenerate in the third position for some codons to increase the chances of primer hybridization to the natural murine DNA codons and also included the unique restriction site, NsiI, for the light chain variable region forward primer and the unique restriction site, MluI, for the heavy chain variable region forward primer to facilitate ligation to the 3' end of the STII element in the vector, pchimFab. The reverse amplification primers were designed to anneal with the murine DNA sequence corresponding to a portion of the constant region of the light or heavy chains near the variable/constant junction. The light chain variable region reverse primer contained a unique MunI restriction site and the heavy chain variable region reverse primer contained a unique ApaI restriction site for ligation to the 5' end of either the human IgG1 constant light or IgG1 constant heavy regions in the vector, pchimFab. The polymerase chain reaction using these primer sets yielded DNA fragments of approximately 400 bp and were cloned individually into the vector, pchimFab, to form p6G425VL and p6G425VH. The cDNA inserts were characterized

by DNA sequencing and are presented in the DNA sequence (SEQ ID NO: 34) and amino acid sequence (SEQ ID NO: 35) of Figure 24 (murine light chain variable region) and the DNA sequence (SEQ ID NO: 36) and amino acid sequence (SEQ ID NO: 37) of Figure 25 (murine heavy chain variable region).

F. CONSTRUCTION OF A 6G4.2.5 CHIMERIC FAB VECTOR

[0286] In the initial construct, p6G425VL, the amino acids between the end of the 6G4.2.5 murine light chain variable sequence and the unique cloning site, MunI, in the human IgG1 constant light sequence were of murine origin. These amino acids must match the human IgG1 amino acid sequence to allow proper folding of the chimeric Fab. Two murine amino acids, D115 and S121, differed dramatically from the amino acids found in the loops of the β-strands of the human IgG1 constant domain and were converted to the proper human amino acid residues, V115 and F121, by site-directed mutagenesis using the primers (SEQ ID NOS: 38,39,40) shown in Figure 26. These specific mutations were confirmed by DNA sequencing and the modified plasmid named p6G425VL'. The coding sequence is shown in the DNA sequence (SEQ ID NO: 41) and amino acid sequence (SEQ ID NO: 42) of Figures 27A-27B.

[0287] Likewise, the DNA sequence between the end of the heavy chain variable region and the unique cloning site, ApaI, in the human IgG1 heavy chain constant domain of p6G425VH was reconstructed to change the amino acids in this area from murine to human. This process was facilitated by the discovery of a BstE11 site near the end of the heavy chain variable region. This site and the ApaI site were used for the addition of a synthetic piece of DNA encoding the corresponding IgG human amino acid sequence. The synthetic oligo-nucleotides shown in Figure 26 were designed as complements of one another to allow the formation of a 27 bp piece of ds DNA. The construction was performed as a three-part ligation because the plasmid, p6G425VH, contained an additional BstE11 site within the vector sequence. A 5309 bp fragment of p6G425VH digested with MluI-ApaI was ligated to a 388 bp fragment carrying the 6G4.2.5 heavy chain variable region and a 27 bp synthetic DNA fragment encoding the first six amino acids of the human IgG1 constant region to form the plasmid, p6G425VH'. The insertion of the synthetic piece of DNA was confirmed by DNA sequencing. The coding sequence is shown in the DNA sequence (SEQ ID NO: 43) and amino acid sequence (SEQ ID NO: 44) of Figures 28A-28B.

[0288] The expression plasmid, p6G425chim2, encoding the chimeric Fab of 6G4.2.5 was made by digesting p6G425chimVL' with MluI and ApaI to remove the STII-murine HPC4 heavy chain variable region and replacing it with the MluI-ApaI fragment encoding the STII-murine 6G4.2.5 heavy chain variable region of p6G425chimVH'. The resultant plasmid thus contained the murine-human variable/constant regions of both the light and heavy chains of 6G4.2.5.

[0289] The plasmid p6G425chim2 was deposited on February 10, 1995 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATTC Accession No. 97055.

G. CONSTRUCTION OF HUMANIZED VERSIONS OF ANTI-IL-8 ANTIBODY 6G4.2.5

[0290] The murine cDNA sequence information obtained from the hybridoma cell line, 6G4.2.5, was used to construct recombinant humanized variants of the murine anti-IL-8 antibody. The first humanized variant, F(ab)-1, was made by grafting synthetic DNA oligonucleotide primers encoding the murine CDRs of the heavy and light chains onto a phagemid vector, pEMX1 (Werther *et al.*, J. Immunol, 157: 4986-4995 (1996)), which contains a human 6-subgroup I light chain and a human IgG1 subgroup III heavy chain (Fig. 29). Amino acids comprising the framework of the antibody that were potentially important for maintaining the conformations necessary for high affinity binding to IL-8 by the complementarity-determining regions (CDR) were identified by comparing molecular models of the murine and humanized 6G4.2.5 (F(ab)-1) variable domains using methods described by Carter *et al.*, PNAS 89:4285 (1992) and Eigenbrot, *et. al.*, J. Mol. Biol. 229:969 (1993). Additional humanized framework variants (F(ab) 2-9) were constructed from the information obtained from these models and are presented in Table 2 below. In these variants, the site-directed mutagenesis methods of Kunkel, Proc. Natl. Acad. Sci USA), 82:488 (1985) were utilized to exchange specific human framework residues with their corresponding 6G4.2.5 murine counterparts. Subsequently, the entire coding sequence of each variant was confirmed by DNA sequencing. Expression and purification of each F(ab) variant was performed as previously described by Werther *et. al.*, *supra*, with the exception that hen egg white lysozyme was omitted from the purification protocol. The variant antibodies were analyzed by SDS-PAGE, electrospray mass spectroscopy and amino acid analysis.

Table 4 -

Humanized 6G425 Variants							
					IC50[c]		
					+-----+		
Variant	**Version**	**Template**	**Changes[a]**	**Purpose[b]**	**Mean**	**S.D.**	**N**
F(ab)-1	version 1		CDR Swap		63.0	12.3	4
F(ab)-2	version 2	F(ab)-1	PheH67*Ala*	packaging w/ CDR H2	106.0	17.0	2
F(ab)-3	version 3	F(ab)-1	ArgH71 *Val*	packaging w/CDRs H1, H2	79.8	42.2	4
F(ab)-4	version 6	F(ab)-1	IleH69*Leu*	packaging w/ CDR H2	44.7	9.0	3
F(ab)-5	version 7	F(ab)-1	LeuH78*Ala*	packaging w/CDRs H1, H2	52.7	31.0	9
F(ab)-6	version 8	F(ab)-1	IleH69*Leu* LeuH78*Ala*	combine F (ab)-4 and -5	34.6	6.7	7
F(ab)-7	version 16	F(ab)-6	LeuH80*Val*	packaging w/ CDR H1	38.4	9.1	2
F(ab)-8	version 19	F(ab)-6	ArgH38*Lys*	packaging w/ CDR H2	14.0	5.7	2
F(ab)-9	version I 1	F(ab)-6	GluH6*Gln*	packaging w/ CDR H3	19.0	5.1	7
Chimeric[d]F (ab)					11.4	7.0	13
rhu4D5[e]F (ab)					>200μ M		5

a Amino acid changes made relative to the template used. Murine residues are in bold italics and residue numbering is according to Kabat *et al.*

b Purpose for making changes based upon interactions observed in molecular models of the humanized and murine variable domains.

c nM concentration of variant necessary to inhibit binding of iodinated IL-8 to human neutrophils in the competitive binding assay.

d Chimeric F(ab) is a (F(ab) which carries the murine heavy and light chain variable domains fused to the human light chain k1 constant domain and the human heavy chain subgroup III constant domain I respectively.

e. rhu4D5F(ab) is of the same isotype as the humanized 6G425 F(ab)s and is a humanized anti-HER2 F(ab) and therefore should not bind to IL8.

[0291] The first humanized variant, F(ab)-1, was an unaltered CDR swap in which all the murine CDR amino acids defined by both x-ray crystallography and sequence hypervariability were transferred to the human framework. When the purified F(ab) was tested for its ability to inhibit ^{125}I-IL-8 binding to human neutrophils according to the methods described in Section (B)(1) above, a 5.5 fold reduction in binding affinity was evident as shown in Table 4 above. Subsequent versions of F(ab)-1 were engineered to fashion the 3-dimensional structure of the CDR loops into a more favorable conformation for binding IL-8. The relative affinities of the F(ab) variants determined from competition binding experiments using human neutrophils as described in Section (B)(1) above are presented in Table 4 above. A slight decrease in IL-8 binding (<2 fold) was observed for F(ab)-2-3 while only slight increases in IL-8 binding were noted for F(ab)3-5. Variant F(ab)-6 had the highest increase in affinity for IL-8 (approximately 2 fold), exhibiting an IL-8 binding affinity of 34.6nM compared to the F(ab)-1 IL-8 binding affinity of 63nM. The substitutions of murine Leu for Ile at H69 and murine Ala for Leu at H78 are predicted to influence the packing of CDRs H1 and H2. Further framework substitutions using the F(ab)-6 variant as template were made to bring the binding affinity closer to that of the chimeric F (ab). *In-vitro* binding experiments revealed no change in affinity for F(ab)-7 (38.4nM) but a significant improvement in affinity for F(ab)-8/9 of 14nM and 19 nM, respectively. By analysis of a 3-D computer-generated model of the anti-IL-8 antibody, it was hypothesized that the substitution of murine Lys for Arg at H38 in F(ab)-8 influences CDR-H2 while

a change at H6 of murine Gln for Glu in F(ab)-9 affects CDR-H3. Examination of the human antibody sequences with respect to amino acid variability revealed that the frequency of Arg at residue H38 is >99% whereas residue H6 is either Gln ~20% or Glu ~80% (Kabat *et. al.*, Sequences of Proteins of Immunological Interest 5th Ed. (1991)). Therefore, to reduce the likelihood of causing an immune response to the antibody, F(ab)-9 was chosen over F(ab)-8 for further affinity maturation studies. Variant F(ab)-9 was also tested for its ability to inhibit IL-8-mediated chemotaxis (Fig. 30). This antibody was able to block neutrophil migration induced by wild-type human IL-8, human monomeric IL-8 and Rhesus IL-8 with IC_{50}=s of approximately 12nM, 15nM, and 22nM, respectively, in IL-8 mediated neutrophil chemotaxis inhibition assays performed as described in Section (B)(2) above. The amino acid sequence for variant F (ab)-8 is provided in Fig. 31c. The F(ab)-8 was found to block human and rhesus IL-8-mediated chemotaxis with IC_{50}=s of .12nM and 10nM, respectively, in IL-8 mediated neutrophil chemotaxis inhibition assays performed as described in Section (B)(2) above.

H. CONSTRUCTION OF AN ANTI-IL-8-GENE III FUSION PROTEIN FOR PHAGE DISPLAY AND ALANINE SCANNING MUTAGENESIS

[0292] An expression plasmid, pPh6G4.V11, encoding a fusion protein (heavy chain of the humanized 6G4.2.5 version 11 antibody and the M 13 phage gene-III coat protein) and the light chain of the humanized 6G4.2.5 version 11 antibody was assembled to produce a monovalent display of the anti-IL-8 antibody on phage particles. The construct was made by digesting the plasmid, pFPHX, with EcoRV and ApaI to remove the existing irrelevant antibody coding sequence and replacing it with a 1305bp EcoRV-ApaI fragment from the plasmid, p6G4.V11, encoding the humanized 6G4.2.5 version 11 anti-IL-8 antibody. The translated sequence of the humanized 6G4.2.5 version 11 heavy chain (SEQ ID NO: 52), peptide linker and gene III coat protein (SEQ ID NO: 53) is shown in Fig. 31A. The pFPHX plasmid is a derivative of phGHam-3 which contains an in-frame amber codon (TAG) between the human growth hormone and gene-III DNA coding sequences. When transformed into an amber suppressor strain of E. coli, the codon (TAG) is read as Glutamate producing a growth hormone (hGH)-gene III fusion protein. Likewise, in a normal strain of *E. coli*, the codon (TAG) is read as a stop preventing translational read-through into the gene-III sequence and thus allowing the production of soluble hGH. The pGHam-3 plasmid is described in Methods: A Companion to Methods in Enzymology, 3:205 (1991). The final product, pPh6G4.V11, was used as the template for the alanine scanning mutagenesis of the CDRs and for the construction of randomized CDR libraries of the humanized 6G4.V11 antibody.

I. ALANINE SCANNING MUTAGENESIS OF HUMANIZED ANTIBODY 6G4.2.5 VERSION 11

[0293] The solvent exposed amino acid residues in the CDRs of the humanized anti-IL-8 6G4.2.5 version 11 antibody (h6G4V11) were identified by analysis of a 3-D computer-generated model of the anti-IL-8 antibody. In order to determine which solvent exposed amino acids in the CDRs affect binding to interleukin-8, each of the solvent exposed amino acids was individually changed to alanine, creating a panel of mutant antibodies wherein each mutant contained an alanine substitution at a single solvent exposed residue. The alanine scanning mutagenesis was performed as described by Leong *et. al.*, J. Biol. Chem., 269: 19343 (1994)).

[0294] The IC_{50}'s (relative affinities) of h6G4V11 wt and mutated antibodies were established using a Competition Phage ELISA Assay described by Cunningham *et. al.*, (EMBO J. 13:2508 (1994)) and Lee *et. al.*, (Science 270:1657 (1995)). The assay measures the ability of each antibody to bind IL-8 coated onto a 96-well plate in the presence of various concentrations of free IL-8 (0.2 to 1uM) in solution. The first step of the assay requires that the concentrations of the phage carrying the wild type and mutated antibodies be normalized, allowing a comparison of the relative affinities of each antibody. The normalization was accomplished by titering the phage on the IL-8 coated plates and establishing their EC_{50}. Sulfhydryl coated 96-well binding plates (Corning-Costar; Wilmington, MA) were incubated with a 0. 1mg/ml solution of K64C IL-8 (Lysine 64 is substituted with Cysteine to allow the formation of a disulfide bond between the free thiol group of K64C IL-8 and the sulfhydryl coated plate, which results in the positioning of the IL-8 receptor binding domains towards the solution interface) in phosphate buffered saline (PBS) pH 6.5 containing 1mM EDTA for 1 hour at 25EC followed by three washes with PBS and a final incubation with a solution of PBS containing 1.75mg/ml of L-cysteine-HCl and 0.1M $NaHCO_3$ to block any free reactive sulfhydryl groups on the plate. The plates were washed once more and stored covered at 4EC with 200ul of PBS/well. Phage displaying either the reference antibody, h6G4V11, or the mutant h6G4V11 antibodies were grown and harvested by PEG precipitation. The phage were resuspended in 500ul 10mM Tris-HCl pH 7.5, 1mM EDTA and 100mM NaCl and held at 4EC for no longer than 3 hours. An aliquot of each phage was diluted 4-fold in PBS containing 0.05% Tween-20 (BioRad, Richmond, Ca.) and 0.5% BSA RIA grade (Sigma, St. Louis, Mo.) (PBB) and added to IL-8 coated plates blocked for at least 2 hours at 25EC with 50mg/ml skim milk powder in 25mM Carbonate Buffer pH 9.6. The phage were next serially diluted in 3 fold steps down the plate from well A through H. The plates were incubated for 1 hour at 25EC followed by nine quick washes with PBS containing 0.05% Tween-20 (PBST). The plates were then incubated with a 1:3200 dilution of rabbit anti-phage antibody and a

1:1600 dilution of secondary goat-anti-rabbit Fc HRP-conjugated antibody for 15 minutes at 25EC followed by nine quick washes with PBST. The plates were developed with 80ul/well of 1mg/ml OPD (Sigma, St. Louis, Mo) in Citrate Phosphate buffer pH 5.0 containing 0.015% H_2O_2 for 4 minutes at 25EC and the reaction stopped with the addition of 40ul of 4.5M H_2SO_4. The plates were analyzed at wavelength 8_{492} in a SLT model 340ATTC plate reader (SLT Lab Instruments). The individual EC_{50}=s were determined by analyzing the data using the program Kaleidagraph (Synergy Software, Reading, Pa.) and a 4-parameter fit equation. The phage held at 4EC were then immediately diluted in PBB to achieve a final concentration corresponding to their respective EC_{50} or target OD_{492} for the competition segment of the experiment, and dispensed into a 96 well plate containing 4-fold serial dilutions of soluble IL-8 ranging from 1uM in well A and ending with 0.2uM in well H. Using a 12-channel pipet, 100ul of the phage/IL-8 mixture was transferred to an IL-8 coated 96-well plate and executed as described above. Each sample was done in triplicate - 3 columns/ sample.

Table 5 -

Relative Affinities (IC50) for Alanine-scan Anti-IL-8 6G4V11 CDR Mutants			
CDR	**Amino Acid Residue**	**Avg IC50 (nM)**	**Std Dev**
V11	Reference	11.5	6.4
CDR-L1	S26	6.3	2.9
	Q27	10.2	2.4
	S28	14.2	5.2
	V30	29.1	12.3
	H31	580.3	243.0
	I33	64.2	14.6
	N35	3.3	0.7
	T36	138.0	nd
	Y37	NDB	nd
CDR-L2	K55	24.2	14.9
	V56	15.5	3.8
	S57	12.4	4.0
	N58	17.6	3.7
	R59	nd	nd
CDR-L3	S96	10.8	4.4
	T97	70.6	55.2
	H98	8.0	1.2
	V99	19.6	1.9
CDR-H1	S28	8.6	3.1
	S30	nd	nd
	S31	7.8	2.5
	H32	13.3	5.8
	Y53	48.2	15.8
CDR-H2	Y50	35.6	13.0
	D52	13.3	7.5
	S53	6.0	3.4

Table 5 - (continued)

Relative Affinities (IC50) for Alanine-scan Anti-IL-8 6G4V11 CDR Mutants			
CDR	Amino Acid Residue	Avg IC50 (nM)	Std Dev
	N54	96.0	5.8
	E56	15.8	4.5
	T57	8.4	1.6
	T58	11.3	1.8
	Y59	9.1	3.7
	Q61	12.6	6.4
	K64	18.5	12.1
CDR-H3	D96	NDB	nd
	Y97	NDB	nd
	R98	36.6	15.3
	Y99	199.5	nd
	N100	278.3	169.4
	D102	159.2	44
	W103	NDB	nd
	F104	NDB	nd
	F105	209.4	72.3
	D106	25.3	21.7

Each sample performed in triplicate/experiment.
NDB = No Detectable Binding /nd = value not determined*
Residue numbering is according to Kabat et al.

[0295] The results of the alanine-scan are summarized in Table 5 above. The alanine substitutions in of many of the mutant antibodies had little or no adverse effects (<3 fold) on the binding affinity for IL-8. Mutants that were found to exhibit no detectable binding of IL-8 (NDB) presumably contained disruptions in the conformational structure of the antibody conferred by crucial structural or buried amino acids in the CDR. Based on the results of the scan, CDR-H3 (heavy chain, 3rd CDR) was identified as the dominant binding epitope for binding IL-8. Alanine substitutions in this CDR resulted in a 3 to >26 fold decrease in binding affinity. The amino acids, Y597, Y599 and D602 are of particular interest because it was determined from the computer generated model of the anti-IL-8 antibody that these residues are solvent exposed and that these residues might participate in hydrogen bonding or charge interactions with IL-8 or other amino acids of the antibody that influence either binding to IL-8 or the conformation of the CDR-H3 loop structure. (See the model depicted in Fig. 32). Unexpected increases in binding affinity (1.8 > 2.7 fold) were noted for S528 and S531 of CDR-H1 and S553 of CDR-H2.

[0296] Surprisingly, a significant increase in binding affinity was observed in the alanine mutant N35A located in CDR-L1 (light chain, 1st CDR). A 3-6 fold increase in affinity was observed compared to the wild-type h6G4V11 antibody. This augmentation of IL-8 binding could be the result of the close proximity of N35A to CDR-H3. The alanine substitution may have imparted a slight change in the conformation of CDR-L1 which alters the packing interaction of neighboring amino acid residues on CDR-H3, thereby tweaking the loop of CDR-H3 into a conformation that facilitates more appropriate contacts with IL-8. Similarly, N35A may also influence the orientation of amino acids in CDR-L1 or its interaction directly with IL-8. Unexpected increases in affinity (-2 fold) were also observed for S26 of CDR-L I and H98 of CDR-L3.

J. CHARACTERIZATION OF HUMANIZED ANTI-IL-8 ANTIBODY 6G4V11N35A

[0297] Soluble 6G4V11N35A Fab antibody was made by transforming an amber non-suppressor strain of *E. coli,* 34B8, with pPh6G4.V11 and growing the culture in low phosphate medium for 24 hours. The periplasmic fraction was

collected and passed over a Hi-Trap Protein-G column (Pharmacia, Piscataway, NJ.) followed by a desalting and concentration step. The protein was analyzed by SDS-PAGE, mass spectrometry and amino acid analysis. The protein had the correct size and amino acid composition (Fig. 35). The 6G4V11N35A Fab was tested for its ability to inhibit ^{125}I-IL-8 binding to human neutrophils and to inhibit IL-8 mediated neutrophil chemotaxis as described in Section (B)(1) and (B)(2) above. As shown in Fig. 33, hybridoma-derived intact murine antibody (6G4 murine mAB), recombinant 6G4 murine-human chimera Fab, recombinant humanized Fab versions 1 and 11, and 6G4V 11N35A Fab were found to inhibit ^{125}I-IL-8 binding to human neutrophils with an average IC_{50} of 5nM, 8nM, 40nM, 10nM and 3nM, respectively. The 6G4V11N35A Fab had at least a 2-fold higher affinity than the 6G4.2.5 chimera Fab and a 3-fold higher affinity than 6G4V11. As shown in Fig. 34, the 6G4V11N35A Fab was found to inhibit IL-8 mediated neutrophil chemotaxis induced by both wild type and monomeric human IL-8, and by two different animal species of IL-8, namely, rabbit and rhesus. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration. The average IC_{50} values were 3nM (wt IL-8), 1 nM (monomeric IL-8), 5nM (Rabbit IL-8), and 10nM (Rhesus IL-8).

K. CONSTRUCTION OF A 6G4V11N35A F(ab')$_2$ LEUCINE ZIPPER

[0298] Production of a F(ab')$_2$ version of the humanized anti-IL-8 6G4V11N35A Fab was accomplished by constructing a fusion protein with the yeast GCN4 leucine zipper. The expression plasmid p6G4V11N35A.F(ab')$_2$ was made by digesting the plasmid p6G425chim2.fab2 with the restriction enzymes bsal and apal to remove the DNA sequence encoding the 6G4.2.5 murine-human chimeric Fab and replacing it with a 2620bp bsal-apal fragment from pPh6G4.V11N35A. The plasmid p6G425chim2.fab2 is a derivative of pS1130 which encodes a fusion protein (the GCN4 leucine zipper fused to the heavy chain of anti-CD18) and the light chain of anti-CD18 antibody. The expression plasmid p6G4V11N35A.F(ab')$_2$ was deposited on February 20, 1996 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, U.S.A. (ATCC) and assigned ATCC Accession No. 97890. A pepsin cleavage site in the hinge region of the antibody facilitates the removal of the leucine zipper leaving the two immunoglobin monomers joined by the cysteines that generate the interchain disulfide bonds. The DNA and protein sequence of the h6G4V11N35A.F(ab')$_2$ are depicted in Figs. 35-37.

[0299] An expression host cell was obtained by transforming E. coli strain 49D6 with p6G4V11N35A.F(ab')$_2$ essentially as described in Section (II)(3)(C) above. The transformed host E. coli 49D6 (p6G4V11N35A.F(ab')$_2$) was deposited on February 20, 1997 at the ATCC and assigned ATCC Accession No. 98332. Transformed host cells were grown in culture, and the 6G4V11N35A F(ab')$_2$ product was harvested from the host cell periplasmic space essentially as described in Section (II)(3)(F) above.

L. CHARACTERIZATION OF THE HUMANIZED 6G4V11N35A F(ab')$_2$ LEUCINE ZIPPER

[0300] The 6G4V11N35A Fab and F(ab')$_2$ were tested for their ability to inhibit ^{125}I-IL-8 binding to neutrophils according to the procedures described in Section (B)(1) above. The displacement curves from a representative binding experiment performed in duplicate is depicted in Fig. 38. Scatchard analysis of this data shows that 6G4V11N35A F (ab')$_2$ inhibited ^{125}I-IL-8 binding to human neutrophils with an average IC_{50} of 0.7 nM (+/- 0.2). This is at least a 7 fold increase in affinity compared to the hybridoma-derived intact murine antibody (average IC_{50} of 5 nM) and at least a 2.8 fold increase in affinity over the Fab version (average IC_{50} of 2 nM).

[0301] The 6G4V 11N35A F(ab')$_2$ was also tested for its ability to inhibit IL-8 mediated neutrophil chemotaxis according to the procedures described in Section (B)(2) above. The results of a representative chemotaxis experiment performed in quadruplicate are depicted in Fig. 39. As shown in Fig. 39, the 6G4V11N35A F(ab')$_2$ inhibited human IL-8 mediated neutrophil chemotaxis. The 6G4V11N35A F(ab')$_2$ exhibited an average IC_{50} value of 1.5nM versus 2.7nM for the 6G4V11N35A Fab, which represents an approximately 2 fold improvement in the antibody's ability to neutralize the effects of IL-8. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migration. Furthermore, the 6G4V11N35A F(ab')$_2$ antibody retained its ability to inhibit IL-8 mediated neutrophil chemotaxis by monomeric IL-8 and by two different animal species of IL-8, namely rabbit and rhesus, in neutrophil chemotaxis experiments conducted as described above. An individual experiment is shown in Fig. 40. The average IC_{50} values were 1nM (monomeric IL-8), 4nM (Rabbit IL-8), and 2.0nM (Rhesus IL-8).

M. RANDOM MUTAGENESIS OF LIGHT CHAIN AMINO ACID (N35A) IN CDR-L1 OF HUMANIZED ANTIBODY 6G4V11

[0302] A 3-fold improvement in the IC_{50} for inhibiting ^{125}I-IL-8 binding to human neutrophils was observed when alanine was substituted for asparagine at position 35 in CDR-L1 (light chain) of the humanized 6G4V11 mAb as described in Section (1) above. This result might be attributed to an improvement in the contact between the antigen-antibody binding interfaces as a consequence of the replacement of a less bulky nonpolar side chain (R-group) that

may have altered the conformation of CDR-L1 or neighboring CDR-H3 (heavy chain) to become more accessible for antigen docking. The acceptance of alanine at position 35 of CDR-L1 suggested that this position contributed to improved affinity and that an assessment of the re-modeling of CDR loops / antigen-binding region(s) by other amino acids at this location was warranted. Selection of an affinity matured version of the humanized 6G4. V11 mAB (Kunkel, T. A., Proc. Natl. Acad. Sci. USA, 82:488 (1995)) was accomplished by randomly mutagenizing position 35 of CDR-L1 and constructing an antibody-phage library. The codon for Asparagine (N) at position 35 of CDR-L1, was targeted for randomization to any of the 20 known amino acids.

[0303] Initially, a stop template, pPh6G4.V11-stop, was made to eliminate contaminating wild-type N35 sequence from the library. This was accomplished by performing site-directed mutagenesis (Muta-Gene Kit, Biorad, Ricmond, CA) of pPH6G4V11 (described in Section (H) above) to replace the codon (AAC) for N35 with a stop codon (TAA) using the primer SL.97.2 (SEQ ID NO:63)(Figure 42). The incorporation of the stop codon was confirmed by DNA sequencing. Subsequently, uracil containing single-stranded DNA derived from *E. coli* CJ236 transformed with the stop template was used to generate an antibody-phage library following the method described by Lowman (Methods in Molecular Biology, 87 Chapter 25: 1-15 (1997). The variants generated from this library were predicted to produce a collection of antibodies containing one of the 20 known amino acids at position N35 in CDR-L1. The amino acid substitutions were accomplished by site-directed mutagenesis using the degenerate oligonucleotide primer (SL.97.3) with the sequence NNS (N = A/G/T/C ; S = G/C;) (SEQ ID NO: 64)(Figure 42). This codon usage should allow for the expression of any of the 20 amino acids - including the amber stop codon (TAG). The collection of antibody-phage variants was transfected into *E. coli* strain XL-1 blue (Stratagene, San Diego, CA) by electroporation and grown at 37°C overnight to amplify the library. Selection of tight binding humanized 6G4V11 Fab's were accomplished by panning the library on IL-8 coated 96-well plates as described in Section (1) above. Prior to panning, the number of phage/library was normalized to 1.1×10^{13} phage/ml (which produces a maximum OD_{270} reading = 1 OD unit) and IL-8 coated plates were incubated with blocking solution (25mN Carbonate buffer containing 50mg/ml skim milk) for 2 hours before the addition of phage (each sort used eight IL-8 coated wells/library). After the blocking and washing steps, every sort began with the addition of 100ul of antibody-phage (titered at 1.1×10^{13} phage/ml) to each of eight IL-8 coated wells followed by an I hour incubation at 25°C. The non-specifically bound antibody-phage were removed by 10 quick washes with PBS-0.05% Tween 20 (PBS-Tween). For sort #1, a low stringency wash (100ul PBS-Tween/well for 10 minutes at 25°C) was employed to capture the small proportion of tight binding antibody-phage bound to the immobilized IL-8. The antibody-phage variants specifically bound to IL-8 were eluted with 100ul/well of 200mM Glycine pH 2.0 for 5 minutes at 25°C. The eluted antibody-phage variants from the 8 wells were then pooled and neutralized with 1M Tris-HCl pH 8.0 (1/3 the elution volume). The phage were titered and propagated as described in Section (I) above. The stringency of the washes were successively increased with each round of panning depending upon the percent recovery of phage at the end of a sort. The wash conditions were as follows: sort #2 (4 x 15 minute intervals; total time = 60 minutes) and sort #3 (either #3a: 8 x 15 minute intervals or #3b: 12 x 10 minute intervals; total time = 120 minutes). The total number of phage recovered was progressively reduced after each sort suggesting that non- or weak- binders were being selected against. The recovery of the negative control (the antibody-phage stop variant) was constant throughout the panning (approximately 0.000 1 to 0.0000 1 percent).

[0304] Eighteen random variants from sort #3 were analyzed by DNA sequencing to look for an amino acid consensus at position 35 of CDR-L1. The data presented in Figure 43A showed that Glycine occupied position 35 in 33% of the variants sequenced. However, after correcting for the number of NNS codon combinations/amino acid, the frequency of Glycine was reduced to 16.6%. Glutamic Acid was represented with the highest frequency (22%) followed by Aspartic Acid and Glycine (16.6%). The frequencies of recovery of the wild-type Asparagine and substituted Alanine were only 5.6%. Interestingly, the high frequency of Glycine may suggest that a much wider range of conformations might be allowed for the loop of CDR-L1 which may be attributed to the reduction in steric hindrance of bond angle (ϕ-ψ) pairing as a result of the single hydrogen atom as the side chain. Conversely, Glutamic Acid at position 35 might restrict the flexibility of the loop by imposing less freedom of rotation imposed by the more rigid and bulky charged polar side chain.

[0305] Soluble Fab's of the affinity marured variants (N35G, N35D, N35E and N35A) were made as described in Section (J) above for evaluating their ability to block IL-8 binding. As shown in Figure 43B, variants N35A, N35D, N35E and N35G were found to inhibit ^{125}I-IL-8 binding to human neutrophils with an approximate IC_{50} of 0.2nM, 0.9nM, 0.1nM and 3.0nM, respectively. All of the affinity matured variants showed an improvement in binding IL-8 ranging from 3 - 100 fold compared to the humanized 6G4V 11 mAb. The affinity-matured variant, 6G4V 11N35E, was 2-fold more potent in blocking IL-8 binding to human neutrophils than the alanine-scan variant, 6G4V11N35A. Equilibrium and kinetic measurements of variants 6G4V11N35A and 6G4V11N35E were determined using KinEXA™ automated immunoassay system (Sapidyne Instruments Inc., Idaho City, ID) as described by Blake et al., J. Biol. Chem. 271: 27677 (1996). The procedure for preparing the antigen-coated particles was modified as follows: 1 ml of activated agarose beads (Reacti-Gel 6X; Pierce, Rockford, IL) were coated with antigen in 50mM Carbonate buffer pH 9.6 containing 20ug/ml of human IL-8 and incubated with gentle agitation on a rocker overnight at 25°C. The IL-8 coated beads were then washed twice with 1M Tris-HCl pH 7.5 to inactivate any unreactive groups on the beads and blocked with Super-

block (Pierce, Rockford, IL) for I hour at 25C to reduce non-specific binding. The beads were resuspended in assay buffer (0.1% bovine serum albumin in PBS) to a final volume of 30 ml. A 550ul aliquot of the IL-8 coated bead suspension was used each time to pack a fresh 4mm high column in the KinEXA observation cell. The amount of unbound antibody from the antibody-antigen mixtures captured by the IL-8-coated beads in both the equilibrium and kinetic experiments was quantified using a fluorescently labeled secondary antibody. Murine 6G4.2.5 was detected with a R-PE AffiniPure $F(ab')_2$ goat anti-mouse IgG, Fc fragment specific 2° antibody (Jackson Immuno Research Laboratories, West Grove, PA) and humanized affinity matured N35A (Fab and $F(ab')_2$) and N35E Fab were detected with a R-PE AffiniPure F $(ab')_2$ donkey anti-human IgG (H+L) 2° antibody (Jackson Immunoresearch Laboratories, West Grove, PA) ; both at a 1:1000 dilution.

[0306] Equilibrium measurements were determined by incubating a constant amount of anti-IL-8 antibody (0.005ug/ml) with various concentrations of human IL-8 (0, 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25, 2.5nM). The antibody-antigen mixture was incuabted for 2 hours at 25°C to allow the molecules to reach equilibrium. Subsequently, each sample was passed over a naive IL-8 coated bead pack in the KinEXA observation cell at a flow rate of 0.5ml/minute for a total of 9 minutes/sample. The equilibrium constant (Kd) was calculated using the software provided by Sapidyne Instruments Inc.

[0307] Rates of association (ka) and dissociation (kd) were determined by incubating together a constant amount of antibody and antigen, and measuring the amount of uncomplexed anti-IL-8 bound to the IL-8 coated beads over time. The concentration of antibody used in the kinetic experiments was identical to that used in the equilibrium experiment described above. Generally, the amount of human IL-8 used was the concentration derived from the binding curves of the equilibrium experiment that resulted in 70% inhibition of anti-IL-8 binding to the IL-8 coated beads. Measurements were made every 15 minutes to collect approximately nine data points. The ka was calculated using the software provided by Sapidyne Instruments, Inc. The off rate was determined using the equation: kd = Kd/ka.

[0308] Figure 44 shows the equilibrium constants (Kd) for the affinity matured variants 6G4V11N35E and 6G4V11N35A Fab's were approximately 54pM and 114pM, respectively. The improvement in affinity of 6G4V11N35E Fab for IL-8 can be attributed to a 2-fold faster rate of association (K_{on}) of 4.7x 10^6 for 6G4V11N35E Fab versus 2.0x10^6 for 6G4V11N35A $F(ab')_2$. (The Kd of the 6G4V11N35A F(ab')2 and 6G4V11N35A Fab are similar.) The dissociation rates (K_{off}) were not significantly different. Molecular modeling suggests that substitution of Aspargine with Glutamic Acid might either affect the antibody's interaction with IL-8 directly or indirectly by neutralizing the charge of neighboring residues R98 (CDR-H3) or K50 (CDR-L2) in the CDR's to facilitate contact with IL-8. Another effect might be the formation of a more stable loop conformation for CDR-L1 that could have facilitated more appropriate contacts of other CDR-L 1 loop residues with IL-8. The DNA (SEQ ID NO: 65) and amino acid (SEQ ID NO:62) sequences of p6G4V 11N35E.Fab showing the Asparagine to Glutamic Acid substitution in the light chain are presented in Figure 45.

N. CHARACTERIZATION OF HUMANIZED ANTI-IL-8 VARIANT 6G4V11N35E Fab

[0309] The affinity matured Fab variant, 6G4V11N35E, was tested for its ability to inhibit IL-8 mediated neutrophil chemotaxis as described in Section (B)(2) above. The reuseable 96-well chemotaxis chamber described in Section (B)(2) was replaced with endotoxin-free disposable chemotaxis chambers containing 5-micron PVP-free polycarbonate filters (ChemoTx101-5, Neuro Probe, Inc. Cabin John, MD). As illustrated in Figure 46, variant N35E effectively blocks IL-8 mediated neutrophil chemotaxis induced by a 2nM stimulus of either rabbit or human IL-8. In fact, the level of inhibition at antibody concentrations between 3.7nM - 33nM was not significantly different from the buffer control indicating variant N35E could completely inhibit this response. The IC_{50}'s for both rabbit and human IL-8 were approximately 2.8nM and 1.2nM, respectively. The irrelevant isotype control Fab (4D5) did not inhibit neutrophil migation indicating the results observed for the affinity matured variant, N35E, is IL-8 specific.

O. CONSTRUCTION OF HUMANIZED 6G4V11N35E $F(ab')_2$ LEUCINE ZIPPER

[0310] A $F(ab')_2$ expression plasmid for 6G4V11N35E was constructed using methods similar to those described in Section (K) above. The expression plasmid, p6G4V11N35E.$F(ab')_2$, was made by digesting the plasmid p6G4V 11N35A.$F(ab')_2$ (described in Section (K) above) with the restriction enzymes ApaI and NdeI to isolate a 2805 bp fragment encoding the heavy chain constant domain -GCN4 leucine zipper and ligating it to a 3758 bp ApaI-NdeI fragment of the pPH6G4V11N35E phage display clone (encoding 6G4V11N35E Fab) obtained as described in Section (M) above. The integrity of the entire coding sequence was confirmed by DNA sequencing.

P. CONSTRUCTION OF THE FULL LENGTH HUMANIZED 6G4V11N35A IgG EXPRESSION PLASMID

[0311] The full length IgG_1 version of the humanized anti-IL8 variant 6G4V11N35A was made using a dicistronic DHFR-Intron expression vector (Lucas et al., Nucleic Acids Res.,24: 1774-1779 (1996)) which contained the full length

recombinant murine-human chimera of the 6G4.2.5 anti-IL8 mAb. The expression plasmid encoding the humanized variant 6G4V11N35A was assembled as follows. First an intermediate plasmid (pSL-3) was made to shuttle the sequence encoding the variable heavy chain of humanized anti-IL-8 variant 6G4V11N35A to pRK56G4chim.2Vh - which contains the variable heavy region of the chimeric 6G4.5 anti-IL8 antibody. The vector pRK56G4chim.Vh was digested with PvuII and ApaI to remove the heavy chain variable region of the chimeric antibody and religated with an 80bp PvuII - XhoI synthetic oligonucleotide (encoding Leu4 to Phe29 of 6G4V11N35A) (Fig. 47) and a 291bp XhoI - ApaI fragment from p6G4V11N35A.7 carrying the remainder of the variable heavy chain sequence of 6G4V11N35A to create pSL-3. This intermediate plasmid was used in conjunction with 2 other plasmids, p6G4V11N35A.F(ab')$_2$ and p6G425chim2.choSD, to create the mammalian expression plasmid, p6G4V11N35AchoSD.9 (identified as p6G425V11N35A.choSD in a deposit made on December 16, 1997 with the ATCC and assigned ATCC Accession No. 209552). This expression construct was assembled in a 4-part ligation using the following DNA fragments: a 5,203bp ClaI - BlpI fragment encoding the regulatory elements of the mammalian expression plasmid (p6G425 chim2.choSD), a 451bp ClaI - ApaI fragment containing the heavy chain variable region of the humanized 6G4V11N35A antibody (pSL-3), a 1,921bp ApaI - EcoRV fragment carrying the heavy chain constant region of 6G4V11N35A (p6G425chim2.choSD) and a 554bp EcoRV - BlpI fragment encoding the light chain variable and constant regions of 6G4V11N35A (p6G4V11N35A.F(ab')$_2$). The DNA sequence (SEQ ID NO: 68) of clone p6G4V11N35A.choSD.9 was confirmed by DNA sequencing and is presented in Figure 48.

Q. CONSTRUCTION OF THE FULL LENGTH HUMANIZED 6G4V11N35E IgG EXPRESSION PLASMID

[0312] A mammalian expression vector for the humanized 6G4V11N35E was made by swapping the light chain variable region of 6G4V11N35A with 6G4V11N35E as follows: a 7,566bp EcoRV - BlpI fragment (void of the 554bp fragment encoding the light chain variable region of 6G4V11N35A) from p6G4V11N35A.choSD.9 was ligated to a 554bp EcoRV - BlpI fragment (encoding the light chain variable region of 6G4V11N35E) from pPH6G4 V 11N35E.7. The mutation at position N35 of the light chain of p6G4V11N35E.choSD.10 was confirmed by DNA sequencing.

R. STABLE CHO CELL LINES FOR VARIANTS N35A AND N35E

[0313] For stable expression of the final humanized IgG1 variants (6G4V11N35A and 6G4V11N35E), Chinese hamster ovary (CHO) DP-12 cells were transfected with the above-described dicistronic vectors (p6G4V11N35A.choSD.9 and p6G4V11N35E.choSD.10, respectively) designed to coexpress both heavy and light chains (Lucas et al., Nucleic Acid Res. 24:1774-79 (1996)). Plasmids were introduced into CHO DP12 cells via lipofection and selected for growth in GHT-free medium (Chisholm, V. High efficiency gene transfer in mammalian cells. In: Glover, DM, Hames, BD. *DNA Cloning 4. Mammalian systems.* Oxford Univ. Press, Oxford pp 1-41 (1996)). Approximately 20 unamplified clones were randomly chosen and reseeded into 96 well plates. Relative specific productivity of each colony was monitored using an ELISA to quantitate the full length human IgG accumulated in each well after 3 days and a fluorescent dye, Calcien AM, as a surrogate marker of viable cell number per well. Based on these data, several unamplified clones were chosen for further amplification in the presence of increasing concentrations of methotrexate. Individual clones surviving at 10, 50, and 100 nM methotrexate were chosen and transferred to 96 well plates for productivity screening. One clone for each antibody (clone#1933 aIL8.92 NB 28605/12 for 6G4V11N35A; clone#1934 aIL8.42 NB 28605/14 for 6G4V11N35E), which reproducibly exhibited high specific productivity, was expanded in T-flasks and used to inoculate a spinner culture. After several passages, the suspension-adapted cells were used to inoculate production cultures in GHT-containing, serum-free media supplemented with various hormones and protein hydrolysates. Harvested cell culture fluid containing recombinant humanized anti-IL8 was purified using protein A-Sepharose CL-4B. The purity after this step was approximately 99% . Subsequent purification to homogeneity was carried out using an ion exchange chromatography step. Production titer of the humanized 6G4V11N35E IgG1 antibody after the first round of amplification and 6G4V11N35A IgG1 after the second round of amplification were 250mg/L and 150mg/L, respectively.

S. CHARACTERIZATION OF THE HUMANIZED 6G4V11N35A/E IgG VARIANTS

[0314] The humanized full length IgG variants of 6G4.2.5 were tested for their ability to inhibit ^{125}I-IL-8 binding and to neutralize activation of human neutrophils; the procedures are described in Sections (B)(1) and (B)(2) above. As shown in Figure 49, the full length IgG1 forms of variants 6G4V11N35A and 6G4V11N35E equally inhibited ^{125}I-IL-8 binding to human neutrophils with approximate IC_{50}'s of 0.3nM and 0.5nM, respectively. This represents a 15 - 25 fold improvement in blocking binding of IL-8 compared to the full length murine mAb (IC_{50} = 7.5nM). Similarly, the two anti-IL-8 variants showed equivalent neutralizing capabilities with respect to inhibiting IL-8 mediated human neutrophil chemotaxis (Figures 50A-50B). The IC_{50}'s of 6G4V11N35A IgG1 and 6G4V11N35E IgG1 for human IL-8 were 4.0nM and 6.0nM, respectively, and for rabbit IL-8 were 4.0nM and 2.0nM, respectively. The irrelevant isotype control Fab

(4D5) did not inhibit neutrophil migration.

[0315] The affinity for IL-8 of these variants relative to the murine 6G4.2.5 mAb was determined using KinExA as described in Section (M). Figure 51 shows the equilibrium constant (Kd) for the full length affinity matured variants 6G4V11N35E IgG1 and 6G4V11N35A IgG1 were approximately 49pM and 88pM, respectively. The Kd for 6G4V11N35A IgG1 was determined directly from the kinetic experiment. As reported with their respective Fabs, this improvement in affinity might be attributed to an approximate 2-fold increase in the on-rate of 6G4V 11N35E IgG1 (ka = 3.0×10^6) compared to that of 6G4V11N35A IgGl (ka = 8.7×10^5). In addition, these results were confirmed by a competition radio-immune assay using iodinated human IL-8. 50pM of 6G4V11N35A IgG1 or 6G4V 11N35E IgG1 was incubated for 2 hours at 25°C with 30-50pM of ^{125}I-IL-8 and varying concentrations (0 to 100nM) of unlabeled IL-8. The antibody-antigen mixture was then incubated for I hour at 4C with 10ul of a 70% slurry of Protein-A beads (preblocked with 0.1% BSA). The beads were briefly spun in a microcentrifuge and the supernatant discarded to remove the unbound ^{125}I-IL-8. The amount of ^{125}I-IL-8 specifically bound to the anti-IL-8 antibodies was determined by counting the protein-A pellets in a gamma counter. The approximate Kd values were similar to those determined by KinEXA. The average Kd for 6G4V11N35A IgG1 and 6G4V11N35E IgG1 were 54pM (18-90pM) and 19pM (5-34pM), respectively (Figure 52).

T. CONSTRUCTION OF HUMANIZED 6G4V11N35A/E Fab's FOR MODIFICATION BY POLYETHYLENE GLYCOL

[0316] A Fab' expression vector for 6G4V11N35A was constructed by digesting p6G4V11N35A.F(ab')$_2$ with the restriction enzymes ApaI and NdeI to remove the 2805 bp fragment encoding the human IgG$_1$ constant domain fused with the yeast GCN4 leucine zipper and replacing it with the 2683bp ApaI-NdeI fragment from the plasmid pCDNA.18 described in Eigenbrot et al., Proteins: Struct. Funct. Genet., 18: 49-62 (1994). The pCDNA.18 ApaI-NdeI fragment carries the coding sequence for the human constant IgG1 heavy domain, including the free cysteine in the hinge region that was used to attach the PEG molecule. The 3758bp ApaI-NdeI fragment (encodes the light chain and heavy variable domain of 6G4V11N35A) isolated from p6G4V11N35A.F(ab')$_2$ was ligated to the 2683bp ApaI-NdeI fragment of pCDNA.18 to create p6G4V11N35A.PEG-1. The integrity of the entire coding sequence was confirmed by DNA sequencing. The nucleotide and translated amino acid sequences of heavy chain constant domain with the cysteine in the hinge are presented in Figure 53.

[0317] A Fab' expression plasmid for 6G4V11N35E was made similarly by digesting pPH6G4V11N35E (from Section (O) above) with the restriction enzymes ApaI and NdeI to isolate the 3758bp ApaI-NdeI DNA fragment carrying the intact light chain and heavy variable domain of 6G4V 11N35E and ligating it to the 2683 bp ApaI-NdeI DNA fragment from p6G4V11N35A.PEG-1 to create p6G4V11N35E.PEG-3. The integrity of the entire coding sequence was confirmed by DNA sequencing.

[0318] Anti-IL-8 6G4V11N35A Fab' variant was modified with 20 kD linear methoxy-PEG-maleimide, 30 kD linear methoxy-PEG-maleimide, 40 kD linear methoxy-PEG-maleimide, or 40 kD branched methoxy-PEG-maleimide as described below. All PEG's used were obtained commercially from Shearwater Polymers, Inc.

a. MATERIALS AND METHODS

Fab'-SH Purification

[0319] A Fab'-SH antibody fragment of the affinity matured antibody 6G4V 11N35A was expressed in *E. coli* grown to high cell density in the fermentor as described by Carter *et al., Bio/Technology* 10, 163-167 (1992). Preparation of Fab'-SH fragments was accomplished by protecting the Fab'-SH fragments with 4',4'-dithiodipyridine (PDS), partially purifying the protected Fab'-PDS fragments, deprotect the Fab'-PDS with dithiothreitol (DTT) and finally isolate the free Fab'-SH by using gel permeation chromatography.

Protection of Fab'-SH with PDS

[0320] Fermentation paste samples were dissolved in 3 volumes of 20mM MES, 5mM EDTA, pH 6.0 containing 10.7mg of 4',4'-dithiodipyridine per gram fermentation paste, resulting in a suspension with a pH close to 6.0 The suspension was passed through a homogenizer followed by addition of 5% PEI (w/v), pH 6 to the homogenate to a final concentration of 0.25%. The mixture was then centrifuged to remove solids and the clear supernatant was conditioned to a conductivity of less than 3mS by the addition of cold water.

Partial purification of the Fab'-SH molecule using ion exchange chromatography

[0321] The conditioned supernatant was loaded onto an ABX (Baker) column equilibrated in 20 mM MES, pH 6.0.

The column was washed with the equilibration buffer followed by elution of the Fab'-SH with a 15 column volume linear gradient from 20 mM MES, pH 6.0 to 20 mM MES, 350 mM sodium chloride. The column was monitored by absorbance at 280nm, and the eluate was collected in fractions.

Deprotection of the Fab'-SH antibody fragments with DTT

[0322] The pH of the ABX pool was adjusted to 4.0 by the addition of dilute HCl. The pH adjusted solution was then deprotected by adding DTT to a final concentration of 0.2mM. The solution was incubated for about 30 minutes and then applied to a gel filtration Sephadex G25 column, equilibrated with 15mM sodium phosphate, 25mM MES, pH 4.0. After elution, the pH of the pool was raised to pH 5.5 and immediately flash frozen at -70°C for storage or derivatized with PEG-MAL as described below.

Alternative Fab'-SH Purification

[0323] Alternatively Fab'-SH fragments can be purified using the following procedure. 100 g fermentation paste is thawed in the presence of 200 ml 50 mM acetic acid, pH 2.8, 2 mM EDTA, 1 mM PMSF. After mixing vigorously for 30 min at room temperature, the extract is incubated with 100 mg hen egg white lysozyme. DEAE fast flow resin (approximately 100 mL) is equilibrated with 10 mM MES, pH 5.5, 1 mM EDTA on a sintered glass funnel. The osmotic shock extract containing the Fab'-SH fragment is then filtered through the resin.

[0324] A protein G Sepharose column is equilibrated with 10 mM MES, pH 5.5, 1 mM EDTA and then loaded with the DEAE flow-through sample. The column is washed followed by three 4 column volume washes with 10 mM MES, pH 5.5, 1 mM EDTA. The Fab'-SH antibody fragment containing a free thiol is eluted from the column with 100 mM acetic acid, pH 2.8, 1 mM EDTA. After elution, the pH of the pool is raised to pH 5.5 and immediately flash frozen at -70°C for storage or derivatized with PEG-MAL as described below.

Preparation of Fab'-S-PEG

[0325] The free thiol content of the Fab'-SH preparation obtained as described above was determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) analysis according to the method of Creighton in Protein Structure: A Practical Approach, Creighton, T.E., ed, IRL Press (Oxford, UK: 1990), pp. 155-167. The concentration of free thiol was calculated from the increase on absorbance at 412 nm, using e_{412} = 14,150 cm^{-1} M^{-1} for the thionitrobenzoate anion and a M_r = 48,690 and e_{280} = 1.5 for the Fab'-SH antibody. To the Fab'-SH protein G Sepharose pool, or the deprotected Fab'-SH gel permeation pool, 5 molar equivalents of PEG-MAL were added and the pH was immediately adjusted to pH 6.5 with 10% NaOH.

[0326] The Fab'-S-PEG was purified using a 2.5 x 20 cm cation exchange column (Poros 50-HS). The column was equilibrated with a buffer containing 20 mM MES, pH 5.5. The coupling reaction containing the PEGylated antibody fragment was diluted with deionized water to a conductivity of approximately 2.0 mS. The conditioned coupling reaction was then loaded onto the equilibrated Poros 50 HS column. Unreacted PEG-MAL was washed from the column with 2 column volumes of 20 mM MES, pH 5.5. The Fab'-S-PEG was eluted from the column using a linear gradient from 0 to 400 mM NaCl, in 20 mM MES pH 5.5, over 15 column volumes.

[0327] Alternatively a Bakerbond ABX column can be used to purify the Fab'-S-PEG molecule. The column is equilibrated with 20 mM MES, pH 6.0 (Buffer A). The coupling reaction is diluted with deionized water until the conductivity equaled that of the Buffer A (approximately 2.0 mS) and loaded onto the column. Unreacted PEG-MAL is washed from the column with 2 column volumes of 20 mM MES, pH 6.0. The Fab'-S-PEG is eluted from the column using a linear gradient from 0 to 100 mM $(NH_4)_2SO_4$, in 20 mM MES pH 6.0, over 15 column volumes.

Size Exclusion Chromatography

[0328] The hydrodynamic or effective size of each molecule was determined using a Pharmacia Superose-6 HR 10/30 column (10x300mm) . The mobile phase was 200 mM NaCl, 50 mM sodium phosphate pH 6.0. Flow rate was at 0.5 ml/min and the column was kept at ambient temperature. Absorbance at 280 nm was monitored where PEG contributed little signal. Biorad MW standards containing cyanocobalamin, myoglobin, ovalbumin, IgG, Thyroglobulin monomer and dimer were used to generate a standard curve from which the effective size of the pegylated species was estimated.

b. RESULTS

Size Exclusion Chromatography

[0329] The effective size of each modified species was characterized using size exclusion chromatography. The results are shown in Fig. 60 below. The theoretical molecular weight of the anti-IL8 Fab fragments modified with PEG 5kD, 10kD, 20kD, 30kD, 40kD (linear), 40kD (branched) or 100,000kD is shown along with the apparent molecular weight of the PEGylated fragments obtained by HPLC size exclusion chromatography. When compared to the theoretical molecular weight of the Fab'-S-PEG fragments, the apparent molecular weight (calculated by size exclusion HPLC) increases dramatically by increasing the size of the PEG attached to the fragments. Attachment of a small molecular weight PEG, for example PEG 10,000D only increases the theoretical molecular weight of the PEGylated antibody fragment (59,700 D) by 3 fold to an apparent molecular weight of 180,000D. In contrast attachment of a larger molecular weight PEG for example 100,000D PEG to the antibody fragment increases the theoretical molecular weight of the PEGylated antibody fragment (158,700 D) by 12 fold to an apparent molecular weight of 2,000,000D.

SDS-PAGE

[0330] In Fig. 61, the upper panel shows the size of the anti-IL-8 Fab fragments modified with PEG of molecular weight 5kD (linear), 10kD (linear), 20kD (linear), 30kD (linear), 40kD (linear), 40kD (branched) or 100kD (linear) under reduced conditions. The unmodified Fab is shown in lane 2 from right to left. Both the heavy and light chains of the Fab had a molecular weight of approximately 30kD as determined by PAGE. Each PEGylated fragment sample produced two bands: (1) a first band (attributed to the light chain) exhibiting a molecular weight of 30kD; and (2) a second band (attributed to the heavy chain to which the PEG is attached specifically at the hinge SH) exhibiting increasing molecular weights of 40, 45, 70, 110, 125, 150 and 300kD. This result suggested that PEGylation was specifically restricted to the heavy chain of the Fab's whereas the light chain remained unmodified.

[0331] The lower panel is non-reduced PAGE showing the size of the anti-IL-8 Fab fragments modified with PEG of molecular weight 5kD (linear), 20kD (linear), 30kD (linear), 40kD (linear), 40kD (branched), or 100kD (linear). The PEGylated fragments exhibited molecular weights of approximately 70kD, 115kD, 120kD, 140kD, 200kD and 300kD.

[0332] The SDS PAGE gels confirm that all Fab'-S-PEG molecules were purified to homogeneity and that the molecules differed only with respect to the size of the PEG molecule attached to them.

U. AMINE SPECIFIC PEGYLATION OF ANTI-IL-8 $F(ab')_2$ FRAGMENTS

[0333] Pegylated $F(ab')_2$ species were generated by using large MW or branched PEGs in order to achieve a large effective size with minimal protein modification which might affect activity. Modification involved N-hydroxysuccinamide chemistry which reacts with primary amines (lysines and the N-terminus). To decrease the probability of modifying the N-terminus, which is in close proximity to the CDR region, a reaction pH of 8, rather than the commonly used pH of 7, was employed. At pH 8.0, the amount of the reactive species (charged NH_3^+) would be considerably more for the ε-NH2 group of lysines (pK_a=10.3) than for the α-NH2 group (pK_a of approximately 7) of the amino-terminus. For the linear PEGs, a methoxy-succinimidyl derivative of an NHS-PEG was used because of the significantly longer half-life in solution (17 minutes at 25°C at pH 8.0) compared to the NHS esters of PEGs (which have 5-7 minute half life under the above conditions). By using a PEG that is less prone to hydrolysis, a greater extent of modification is achieved with less PEG. Branched PEGs were used to induce a large increase in effective size of the antibody fragments.

a. MATERIALS

[0334] All PEG reagents were purchased from Shearwater Polymers and stored at -70°C in a desiccator. branched N-hydroxysuccinamide-PEG (PEG2-NHS-40KDa) has a 20 kDa PEG on each of the two branches, methoxy-succinimidyl-propionic acid-PEG (M-SPA-20000) is a linear PEG molecule with 20 kDa PEG. Protein was recombinantly produced in *E. coli* and purified as a $(Fab)'_2$ as described in Sections (K) and (O) above.

b. METHODS

[0335] ***IEX method:*** A J. T. Baker Wide-Pore Carboxy-sulfone (CSX), 5 micron, 7.75 x 100 mm HPLC column was used for fractionation of the different pegylated products, taking advantage of the difference in charge as the lysines are modified. The column was heated at 40°C. A gradient as shown in Table 7 below was used where Buffer A was 25 mM sodium Borate/25 mM sodium phosphate pH 6.0, and Buffer B was I M ammonium sulfate, and Buffer C was 50 mM sodium acetate pH 5.0.

Table 7

Time (min)	*%B*	*%C*	*flow mL/min*
0	10	10	1.5
20	18	7.5	1.5
25	25	7.5	1.5
27	70	3.0	2.5
29	70	3.0	2.5
30	10	10	2.5
33	10	10	2.5

[0336] ***SEC-HPLC***: The hydrodynamic or effective size of each molecule was determined using a Pharmacia Superose-6 HR 10/30 column (10x300mm). The mobile phase was 200 mM NaCl, 50 mM sodium phosphate pH 6.0. Flow rate was at 0.5 ml/min and the column was kept at ambient temperature. Absorbance at 280 nm was monitored where PEG contributed little signal. Biorad MW standards containing cyanocobalamin, myoglobin, ovalbumin, IgG, Thyroglobulin monomer and dimer were used to generate a standard curve from which the effective size of the pegylated species was estimated.

[0337] ***SEC-HPLC-Light Scattering***: For determination of the exact molecular weight, this column was connected to an on-line light scattering detector (Wyatt Minidawn) equipped with three detection angles of 50°, 90°, and 135° C. A refractive index detector (Wyatt) was also placed on-line to determine concentration. All buffers were filtered with Millipore 0.1 μ filters; in addition al 0.02 μ Whatman Anodisc 47 was placed on-line prior to the column.

[0338] The intensity of scattered light is directly proportional to the molecular weight (M) of the scattering species, independent of s hape, according to:

$$M = R_0/K.c$$

where R_0 is the Rayleigh ratio, K is an optical constant relating to the refractive index of the solvent, the wavelength of the incident light, and dn/dc, the differential refractive index between the solvent and the solute with respect to the change in solute concentration, c. The system was calibrated with toluene (R_0 of 1.406×10^{-5} at 632.8 nm); a dn/dc of 0.18, and an extinction coefficient of 1.2 was used. The system had a mass accuracy of ~5%.

[0339] ***SDS-PAGE***: 4-12% Tris-Glycine Novex minigels were used along with the Novex supplied Tris-Glycine running buffers. 10-20 ug of protein was applied in each well and the gels were run in a cold box at 150 mV/gel for 45 minutes. Gels were then stained with colloidal Coomassie Blue (Novex) and then washed with water for a few hours and then preserved and dried in drying buffer (Novex)

[0340] **Preparation of a linear(1)20KDa-(N)-(Fab')2**: A 4 mg/ml solution of anti-IL8 formulated initially in a pH 5.5 buffer was dialyzed overnight against a pH 8.0 sodium phosphate buffer. 5 mL protein was mixed at a molar ratio of 3:1. The reaction was carried out in a 15mL polypropylene Falcon tube and the PEG was added while vortexing the sample at low speed for 5 seconds. It was then placed on a nutator for 30 minutes. The extent of modification was evaluated by SDS-PAGE. The whole 5 ml reaction mixture was injected on the IEX for removal of any unreacted PEG and purification of singly or doubly pegylated species. The above reaction generated a mixture of 50% singly-labeled anti-1L8. The other 50% unreacted anti-IL8 was recycled through the pegylation/purification steps. The pooled pegylated product was dialyzed against a pH 5.5 buffer for in vitro assays and animal PK studies. Endotoxin levels were measured before administration to animals or for the cell based assays. Levels were below 0.5 eu/ml. The fractions were also run on SDS-PAGE to confirm homogeneity. Concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

[0341] Preparation of a branched(1)40KDa-(N)-(Fab')2: A 4 mg/mL solution of anti-IL8 $(Fab')_2$ formulated in a pH 5.5 buffer was dialyzed overnight against a pH 8.0 phosphate buffer. Solid PEG powder was added to 5 mL protein in two aliquots to give a final PEG:protein molar ratio of 6:1. Each solid PEG aliquot was added to the protein in a 15 mL polypropylene Falcon tube while vortexing at low speed for 5 sec, and then placing the sample on a nutator for 15 minutes. The extent of modification was evaluated by SDS-PAGE using a 4-12% Tris-Glycine (Novex) gel and stained with colloidal Coomasie blue (Novex). The 5 mL PEG-protein mixture was injected on the ion exchange column for removal of any unreacted PEG. The above reaction generated a mixture of unreacted (37%), singly-labelled (45%), doubly and triply-labeled (18%) species. These were the optimal conditions for obtaining the greatest recovery of the protein with only 1 PEG per antibody rather than the higher molecular weight adducts. The unmodified anti-IL8 was recycled. The pegylated products were separated and fractionated in falcon tubes and then dialyzed against a pH 5.5

buffer for assays and animal PK studies. Endotoxin levels were below 0.5 eu/ml. The fractions were also run on SDS-PAGE to confirm homogeneity. The concentration of the final product was assessed by absorbance at 280 nm using an extinction coefficient of 1.34, as well as by amino acid analysis.

[0342] **Preparation of branched(2)-40KDa-(N)(Fab')2**: This molecule was most efficiently made by adding three times in 15 minute intervals a 3:1 molar ratio of PEG to the already modified branched(1)-40KDa-(N)-(Fab')2. The molecule was purified on IEX as 50% branched(2)-40KDa-(N)-(Fab')2. The unmodified molecule was recycled until ~20 mg protein was isolated for animal PK studies. The product was characterized by SEC-light scattering and SDS-PAGE.

c. RESULTS

[0343] PEGs increased the hydrodynamic or effective size of the product significantly as determined by gel filtration (SEC-HPLC). Figure 62 shows the SEC profile of the pegylated F(ab')$_2$ species with UV detection at 280 nm. The hydrodynamic size of each molecule was estimated by reference to the standard MW calibrators. As summarized in Figure 62, the increase in the effective size of (Fab')$_2$ was about 7-fold by adding one linear 20 kDa PEG molecule and about 11-fold by adding one branched ("Br(1)") 40 kDa PEG molecule, and somewhat more with addition of two branched ("Br(2)") PEG molecules.

[0344] Light scattering detection gave the exact molecular weight of the products and confirmed the extent of modification (Figure 63). The homogeneity of the purified material was shown by SDS-PAGE (Figure 64). Underivatized F(ab')$_2$ migrated as a 120 kDa species, the linear(1)20KD-(N)-F(ab')$_2$ migrated as a band at 220kDa, the Br(1)-40KD (N)-F(ab')$_2$ migrated as one major band at 400 kDa, and the Br(2)-40KD-(N)-F(ab')$_2$ migrated as a major band at around 500 kDa. The proteins appeared somewhat larger than their absolute MW due to the steric effect of PEG.

V. IN VITRO ACTIVITY CHARACTERIZATION OF PEG MODIFIED Fab' FRAGMENTS OF 6G4V 11N35A (MALEIMIDE CHEMICAL COUPLING METHOD)

[0345] Anti-IL-8 6G4V 11N35A Fab' variants modified with 5-40kD linear PEG molecules and a 40kD branched PEG molecule were tested for their ability to inhibit both IL-8 binding and activation of human neutrophils; the procedures were described in Sections (B)(1), (B)(2) and (B)(3) above. The binding curves and IC$_{50}$'s for PEG-maleimide modified 6G4V 11N35A Fab' molecules are presented in Figures 54A-54C. The IC$_{50}$ of the 5kD pegylated Fab' (350pM) and the average IC$_{50}$ of the Fab control (366pM) were not significantly different, suggesting that the addition of a 5kD MW PEG did not affect the binding of IL-8 to the modified Fab' (Figure 54A). However, a decrease in the binding of IL-8 to the 10kD and 20kD pegylated Fab' molecules was observed as depicted by the progressively higher IC$_{50}$'s (537pM and 732pM, respectively) compared to the average IC$_{50}$ of the native Fab. These values represent only a minimal loss of binding activity (between 1.5- and 2.0-fold). A less pronounced difference in IL-8 binding was observed for the 30kD and 40kD linear PEG antibodies (Figure 54B). The IC$_{50}$'s were 624pM and 1.1nM, respectively, compared to the 802pM value of the Fab control. The 40kD branched PEG Fab' showed the largest decrease in IL-8 binding (2.5 fold) relative to the native Fab (Figure 54C). Nevertheless, the reduction in binding of IL-8 by these pegylated Fab's is minimal.

[0346] The ability of the pegylated antibodies to block IL-8 mediated activation of human neutrophils was demonstrated using the PMN chemotaxis (according to the method described in Section B(2) above) and β-glucuronidase release (according to the method described in Lowman et al., J. Biol. Chem., 271: 14344 (1996)) assays. The IC$_{50}$'s for blocking IL-8 mediated chemotaxis are shown in Figures 55A-55C. The 5-20kD linear pegylated Fab' antibodies were able to block IL-8 mediated chemotaxis within 2-3 fold of the unpegylated Fab control (Figure 55A). This difference is not significant because the inherent variation can be up to 2 fold for this type of assay. However, a significant difference was detected for the 30kD and 40kD linear pegylated Fab' antibodies as illustrated by the higher IC$_{50}$'s of the 30kD linear PEG-Fab' (2.5nM) and 40kD linear PEG-Fab' (3.7nM) compared to the Fab control (0.8nM) (Figure 55B). The ability of the 40kD branched PEG Fab' molecule to block IL-8 mediated chemotaxis was similar to that of the 40kD linear PEG Fab' (Figure 55C). At most, the ability of the pegylated Fab' antibodies to block IL-8 mediated chemotaxis was only reduced 2-3 fold. Furthermore, release of β-glucuronidase from the granules of neutrophils was used as another criteria for assessing IL-8 mediated activation of human PMNs. Figure 56A (depicting results obtained with 5 kD, 10 kD and 20 kD linear PEGs), Figure 56B (depicting results obtained with 30 kD and 40 kD linear PEGs), and Figure 56C (depicting results obtained with 40 kD branched PEG) show that all the pegylated Fab' antibodies were able to inhibit IL-8 mediated release of β-glucuronidase as well as or better than the unpegylated Fab control. The data collectively shows that the pegylated Fab' variants are biological active and are capable of inhibiting high amounts of exogenous IL-8 in in-vitro assays using human neutrophils.

W. IN VITRO ACTIVITY CHARACTERIZATION OF PEG MODIFIED $F(ab')_2$ FRAGMENTS OF 6G4V11N35A (SUCCINIMIDYL CHEMICAL COUPLING METHOD)

[0347] The anti-IL-8 variant 6G4V11N35A $F(ab')_2$ modified with (a) a single 20kD linear PEG molecule per $F(ab')_2$, (b) a single 40kD branched PEG molecule per $F(ab')_2$, (c) with three, four, or five 20 kD linear PEG molecules per $F(ab')_2$ (a mixture of: (1) species having three 20 kD linear PEG molecules per $F(ab')_2$; (2) species having four 20 kD linear PEG molecules per $F(ab')_2$; and (3) species having five 20 kD linear PEG molecules per $F(ab')_2$; denoted as "20 kD linear PEG (3,4,5) $F(ab')_2$"), or (d) with two 40kD branched PEG molecules per $F(ab')_2$ (denoted as "40 kD branch PEG (2) $F(ab')_2$"), were tested for their ability to inhibit ^{125}I-IL-8 binding and to neutralize activation of human neutrophils. The procedures used are described in Sections (B)(1), (B)(2) and (B)(3) above. The binding curves for pegylated $F(ab')_2$ variants are shown in Figures 57A-57B. No significant differences were observed amongst the $F(ab')_2$ control, the single 20kD linear PEG-modified $F(ab')_2$, and the single 40kD branched PEG-modified $F(ab')_2$ (Figure 57A). However, the $F(ab')_2$ variants containing multiple PEG molecules showed a slight reduction (less than 2-fold) in their ability to bind IL-8. The IC_{50}'s of the 20kD linear PEG (3,4,5) $F(ab')_2$ and 40kD branch PEG (2) $F(ab')_2$ variants were 437pM and 510pM, respectively, compared to 349pM of the $F(ab')_2$ control (Figure 57B).

[0348] The ability of these pegylated $F(ab')_2$ variants to block IL-8 mediated neutrophil chemotaxis is presented in Figures 58A-58B. Consistent with the PMN binding data, the single linear and branched PEG $F(ab')_2$ variants were able to block IL-8 mediated chemotaxis similar to the unpegylated $F(ab')_2$ control (Figure 58A). The ability of the 40kD branch PEG (2) $F(ab')_2$ variant to inhibit PMN chemotaxis was identical to the control $F(ab')_2$ while the 20kD linear PEG (3,4,5) $F(ab')_2$ mixture was able to inhibit within 3-fold of the control antibody (Figure 58B).

[0349] Shown in Figures 59A and 59B are the results of the β-glucuronidase release assay which is a measure of degranulation by IL-8 stimulated human neutrophils. The single 20kD linear PEG-modified $F(ab')_2$ and the single 40kD branched PEG-modified $F(ab')_2$ variants were able to inhibit release of β-glucuronidase as well as the $F(ab')_2$ control (Figure 59A). The 40kD branch PEG (2) $F(ab')_2$ inhibited this response within 2-fold of the $F(ab')_2$ control (Figure 59B). The 20kD linear PEG (3,4,5) molecule was not tested. Overall, the $F(ab')_2$ pegylated anti-IL-8 antibodies were biologically active and effectively prevented IL-8 binding to human neutrophils and the signaling events leading to cellular activation.

X. PHARMACOKINETIC AND SAFETY STUDY OF EIGHT CONSTRUCTS OF PEGYLATED ANTI-IL-8 (HUMANIZED) F(AB')2 AND FAB' FRAGMENTS IN NORMAL RABBITS FOLLOWING INTRAVENOUS ADMINISTRATION

[0350] The objective of this study was to evaluate the effect of pegylation on the pharmacokinetics and safety of six pegylated humanized anti-IL-8 constructs (pegylated 6G4V 11N35A.Fab' and pegylated 6G4V11N35A.$F(ab')_2$ obtained as described in Sections (T) and (U) above) relative to the non-pegylated fragments in normal rabbits. Eight groups of two/three male rabbits received equivalent protein amounts of pegylated 6G4V11N35A.Fab' or pegylated 6G4V11N35A.$F(ab')_2$ constructs (2 mg/kg) via a single intravenous (IV) bolus dose of one anti-IL8 construct. Serum samples were collected according to the schedule shown in Table 8 below and analyzed for anti-IL8 protein concentrations and antibody formation against anti-IL8 constructs by ELISA.

Table 8

Group No.	Dose level/ Route	Material	Blood Collection
1	2 mg/kg (protein conc.) IV bolus	Fab' control	0,5,30 min; 1,2,3,4,6,8,10, 14,20,24,360 hr
2		linear(1)20K(s)Fab'	
3		linear(1)40K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216, 264,336,360 hr
4		branched(1)40K(N)F(ab')$_2$	
5		F(ab')$_2$ control	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,52,56,336 hr
6		branched(2)40K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216,264,3 36 hr; Day 17,21, 25
7		branched(2)40K(N)F(ab')$_2$	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,144,192,240 hr; Day 13, 16, 20, 23
8		linear(1)30K(s)Fab'	0,5,30 min; 1,2,4,6,8,10,12, 24,28,32,48,72,96,168,216,264,3 36 hr; Day 17,21,25

a. METHODS

[0351] Three male New Zealand White (NZW) rabbits per group (with exception to Group 7, n=2) received an equivalent amount of 6G4V11N35A protein (Fab' or F(ab')$_2$) construct at 2 mg/kg via an IV bolus dose in a marginal ear vein. Amino acid composition analysis and absorbance at 280 nm using extinction coefficients of 1.26 for 6G4V11N35A Fab' constructs and 1.34 for 6G4V11N35A F(ab')$_2$ constructs were performed to determine the protein concentration. Whole blood samples were collected via an ear artery cannulation (ear opposing dosing ear) at the above time points. Samples were harvested for serum and assayed for free 6G4V11N35A Fab' or F(ab')$_2$ constructs using an IL-8 Binding ELISA. Assays were conducted throughout the study as samples became available. All animals were sacrificed following the last blood draw, and necropsies were performed on all animals in Groups 1, 4-8. Due to the development of antibodies against the 6G4V11N35A constructs, non-compartmental pharmacokinetic analysis was conducted on concentration versus time data only up to 168 hours.

b. RESULTS

[0352] In four animals (Animals B, P, Q, V), interference to rabbit serum in the ELISA assay was detected (i.e. measurable concentrations of anti-IL8 antibodies at pre-dose). However, because these values were at insignificant levels and did not effect the pharmacokinetic analysis, the data were not corrected for this interference.

[0353] One animal (Animal G; Group 3) was exsanguinated before the termination of the study and was excluded from the pharmacokinetic analysis. At 4 hours, the animal showed signs of a stroke that was not believed to be drug related, as this can occur in rabbits following blood draws via ear artery cannulation.

[0354] The mean concentration-time profiles of the eight anti-IL8 constructs in normal rabbits are depicted in Fig. 65, and the pharmacokinetic parameters for the eight constructs are summarized in Table 9 below. Significant antibodies to the anti-IL-8 constructs were present at Day 13/14 in all dose groups except Group 1 (Fab' control).

Table 9. Pharmacokinetic parameters.

Molecule	Fab'					$F(ab')_2$		
Group No.	1	2	8	3	6	5	4	7
PEG structure	—	linear	linear	linear	branched	—	branched	branched
Number of PEGs	—	1	1	1	1	—	1	2
PEG MW	—	20K	30K	40K	40K	—	40K	40K
Dose (mg/kg)	2	2	2	2	2	2	2	2
V_c (mL/kg)[a]	58±3	36±3	35±1	34	44±1	45±5	36±1	32
V_{ss} (mL/kg)[b]	68±8	80±8	110±15	79	88±21	59±4	50±3	52
Cmax (μg/mL)[c]	35±1	58±3	57±1	60	45±1	45±6	56±2	62
Tmax (min)[d]	5	5	5	5	5	5	5	5
$t_{1/2}$ term (hr)[e]	3.0±0.9	44±2	43±7	50	105±11	8.5±2.1	45±3	48
AUC_{0-} (hr·μg/mL)[f]	18±3	80±74	910±140	1600	3400±1300	140±3	2200±77	2500
CL (mL/hr/kg)[g]	110±17	2.5±0.2	2.2±0.4	1.3	0.63±0.20	14±0	0.92±0.03	0.83
MRT (hr)[h]	0.61±0.15	32±2	45±9	63	140±18	4.2±0.3	55±3	64
No. of Animals	3	3	3	2	3	3	3	2

[a] Initial volume of distribution.
[b] Volume of distribution at steady state.
[c] Observed maximum concentration.
[d] Observed time to Cmax.
[e] $t_{1/2}$ term= half-life associated with the terminal phase of the concentration vs. time profile.
[f] Area under the concentration versus time curve (extrapolated to infinity).

[g] CL= serum clearance.
[h] MRT= Mean residence time.
The initial volume of distribution approximated the plasma volume for both the Fab' and $F(ab')_2$.

Pegylation decreased serum CL of anti-IL8 fragments and extended both the terminal half-life and MRT as shown in Table 10 below.

Table 10.

Fold decrease/increase in clearance, terminal half-life & MRT of pegylated anti-IL8 fragments.								
anti-IL8 fragment	Fab'					$F(ab')_2$		
Group No.	1	2	8	3	6	5	4	7
PEG structure No. of PEGs PEG MW	- - -	linear 1 20K	linear 1 30K	linear 1 40K	bran. 1 40K	- - -	bran. 1 40K	bran. 2 40K
CL : mean (mL/hr/kg) fold decrease	110 1	2.5 46	2.2 51	1.3 90	0.63 180	14 1	0.92 15	0.83 17
t1/2 term : mean (hr) fold increase	3.0 1	44 14	43 14	50 17	110 35	8.5 1	45 5.3	48 5.7
MRT: mean (hr) fold increase	0.61 1	32 53	45 73	63 100	140 240	4.2 1	55 13	64 15

[0355] For the pegylated anti-IL8 Fab' fragments, CL decreased by 46 to 180-fold. Terminal half-life and MRT increased 14 to 35-fold and 53 to 240-fold, respectively. For pegylated anti-IL8 $F(ab')_2$ molecules, CL decreased 15 to 17-fold with pegylation, and terminal half-life and MRT increased by greater than 5-fold and 13-fold, respectively. The changes in these parameters increased for both pegylated Fab' and $F(ab')_2$ molecules with increasing PEG molecular weight and approached the values of the full-length anti-IL8 (terminal half-life of 74 hours, MRT of 99 hours and CL of 0.47 mL/hr/kg). In comparing the branched(1)40K Fab' (Group 6) and branched(1)40K $F(ab')_2$ (Group 4), unexpected pharmacokinetics were observed. The pegylated Fab' molecule appeared to remain in the serum longer than the pegylated $F(ab')_2$ (see Figure 66). The mean CL of branched(1)40K Fab' was 0.63 mL/hr/kg, but a higher CL was observed for branched(1)40kD $F(ab')_2$ (CL 0.92 mL/hr/kg). The terminal half-life, likewise, was longer for the Fab' than the $F(ab')_2$ pegylated molecule (110 vs 45 hours).

[0356] The pharmacokinetic data demonstrated that pegylation decreased CL and increased terminal t1/2 and MRT of anti-IL8 fragments (Fab' and $F(ab')_2$) to approach that of the full-length anti-IL8. Clearance was decreased with pegylation 46 to 180-fold for the Fab' and approximately 16-fold for the $F(ab')_2$. The terminal half-life of the Fab' anti-IL8 fragment was increased by 14 to 35-fold and approximately 5-fold for the $F(ab')_2$ anti-IL8. MRT, likewise, were extended by 53 to 240-fold for the Fab' and approximately 14-fold for the $F(ab')_2$. The branched(1) 40kD Fab' had a longer terminal half-life and lower clearance compared to the branched(1) 40kD $F(ab')_2$.

Y. <u>IN VIVO EFFICACY TESTING OF ANTI-IL-8 ANTIBODY REAGENTS IN RABBIT MODEL OF ISCHEMIA/ REPERFUSION AND ACID ASPIRATION-INDUCED ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)</u>

[0357] Full length murine anti-rabbit IL-8 monoclonal antibody 6G4.2.5, 40 kD branched PEG-6G4V11N35A Fab', and control antibody (anti-HIV gp120 monoclonal antibody 9E3.1F10) were tested in a rabbit ARDS model. The animals were weighed and anaesthetized by intramuscular injection of ketamine (50 mg/kg body weight), xylazine (5 mg/kg body weight), and acepromazine (0.75 mg/kg body weight). A second dose (20% of the first dosage) was given 1M 15 minutes before removal of vascular clip, and third dose (60% of the first dosage) was given at tracheotomy. Intra-arterial catheter (22G, 1 in. Angiocath) and intra-venous catheter (24G, 1 in. aneiocath) were be placed in the ear central artery and posterior marginal ear vein for blood samplings (arterial blood gases and CBC) and anti-IL-8 and fluid administration, respectively. The anaesthetized animals were transferred in a supine position to an operating tray; the abdominal area was shaved and prepared for surgery. Via a midline laparotomy, the superior mesenteric artery (SMA) was isolated and a microvascular arterial clip applied at the aortic origin. Before the temporary closure of the abdomen using 9 mm wound clip (Autoclip, Baxter), 15 ml of normal saline was given intraperitoneally as fluid supplement. After 110 minutes of intestinal ischemia, the abdominal incision was reopened and the arterial clip was released to allow reperfusion. Before closure, 5 ml of normal saline was given intraperitoneally for fluid replacement. The laparotomy incision was closed in two layers and the animals allowed to awaken.

[0358] After surgery, the animals were placed on a heating pad (38°C) and continuously monitored for up to 6 hours post reperfusion and lactated Ringer's 8-12 ml/kg/hr IV was given as fluid supplement.

[0359] At 22-24 hr post-reperfusion, a tracheotomy was performed under anesthesia. Normal physiologic saline was diluted 1:3 with water and adjusted to pH 1.5 (adjusted by using IN HCL); 3 ml/kg body weight was then instilled intratracheally. Rectal temperature was maintained at 37 +/- 1 degree C using a homeothermic heat therapy pad (K-Mod

II, Baxter). Fluid supplements (LRS) at a rate of 5 ml/kg/hour IV were given. Blood gases were monitored every hour. The rabbits were returned to the cage after 6 hr of continuous monitoring.

[0360] Just prior to aspiration, animals were treated with saline, the control monoclonal antibody (anti-HIV gp-120 IgG 9E3.IF10), the full length murine anti-rabbit IL8 (6g4.2.5 murine IgG2a anti-rabbit IL8) or the pegylated 6G4V11N35A Fab' (6G4V 1N35A Fab' modified with 40kD branched PEG-maleimide as described in Section T above, denoted as "40 kD branched PEG-6G4V11N35A Fab' "). Data from saline or control antibody treated animals was combined and presented as "Control". Arterial blood gases and A-a PO2 gradient measurements were taken daily, and IV fluid supplementation was performed daily. A-a PO2 gradient was measured at 96 hr of reperfusion. The A-a PO2 gradient was calculated as:

$$\text{A-a PO2} = [\text{FIO2}(\text{PB} - \text{PH2O}) - (\text{PaCO2/RQ})] - \text{PaO2}.$$

[0361] PaO2/FiO2 ratios were measured at 24hr and 48hr in room air and 100% oxygen.

[0362] After the final A-a PO2 gradient measurement, the animals were anesthetized with Nembutal 100mg/kg i.v. and the animals were euthanized by transecting the abdominal aorta in order to reduce red blood cell contamination of bronchoalveolar lavage fluid (BAL). The lungs were removed en bloc. The entire lung was weighed and then lavaged with an intratracheal tube (Hi-Lo tracheal tube, 3mm) using 30 ml of HBSS and lidocain. Total and differential leukocyte counts in the BAL were determined. Lesions/changes were verified by histological examination of each lobe of the right lung of each animal.

[0363] The gross lung weight, total leukocyte and polymorphonuclear cell counts in BAL, and PaO2/FiO2 data obtained are depicted in Figs. 67, 68 and 69, respectively. Treatment with 40 kD branched PEG-6G4V11N35A Fab' exhibited no effect on the biological parameters measured in the model as compared to the "Control" group. However, the data do not contradict the pharmacokinetic analysis or the in vitro activity analysis for the 40 kD branched PEG-6G4V11N35A Fab' presented in Sections (V) and (X) above. In addition, these data do not contradict the ability of the 40 kD branched PEG-6G4V11N35A Fab' to reach and act on disease effector targets in circulation or other tissues.

[0364] The following biological materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Material	ATCC Accession No.	Deposit Date
hybridoma cell line 5.12.14	HB 11553	February 15, 1993
hybridoma cell line 6G4.2.5	HB 11722	September 28, 1994
pantilL-8.2, E. coli strain 294 mm	97056	February 10, 1995
p6G425chim2, E. coli strain 294 mm	97055	February 10, 1995
p6G4V11N35A.F(ab')$_2$	97890	February 20, 1997
E. coli strain 49D6(p6G4V 11N35A.F(ab')$_2$)	98332	February 20, 1997
p6G425V11N35A.choSD	209552	December 16, 1997
clone#1933 aIL8.92 NB 28605/12	CRL-12444	December 11, 1997
clone#1934 aIL8.42 NB 28605/14	CRL-12445	December 11, 1997

[0365] These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable deposit for 30 years from the date of deposit. These cell lines will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the cell lines to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

[0366] The assignee of the present application has agreed that if the deposited cell lines should be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a specimen of the same cell line. Availability of the deposited cell lines is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Claims

1. A conjugate consisting essentially of one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, said antibody fragments comprising the antigen binding site or variable region of the intact antibody but free of the constant heavy chain domains of the Fc region of the intact antibody, wherein the antibody fragment comprises a heavy chain and a light chain derived from a parental antibody, wherein in the parental antibody the heavy and light chains are covalently linked by a disulfide bond between a cysteine residue in the light chain and a cysteine residue in the heavy chain, wherein in the antibody fragment the cysteine residue in the light or heavy chain is substituted with another amino acid and the cysteine residue in the opposite chain is covalently linked to a nonproteinaceous polymer molecule, and wherein the apparent size of the conjugate is at least about 500 kD.

2. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 800 kD.

3. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 1,400 kD.

4. The conjugate of claim 1, wherein the apparent size of the conjugate is at least about 1,800 kD.

5. The conjugate of claim 1, wherein the apparent size of the conjugate is at least 8 fold greater than the apparent size of the antibody fragment.

6. The conjugate of claim 5, wherein the apparent size of the conjugate is at least 15 fold greater than the apparent size of the antibody fragment.

7. The conjugate of claim 6, wherein the apparent size of the conjugate is at least 25 fold greater than the apparent size of the antibody fragment.

8. The conjugate of claim 1, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is selected from the group consisting of Fab, Fab', Fab'-SH, Fv, scFv and $F(ab')_2$.

9. The conjugate of claim 8 wherein the antibody fragment is $F(ab')_2$.

10. The conjugate of claim 1 wherein the antibody fragment is covalently attached to no more than 10 nonproteinaceous polymer molecules.

11. The conjugate of claim 10 wherein the antibody fragment is covalently attached to no more than 5 nonproteinaceous polymer molecules.

12. The conjugate of claim 11 wherein the antibody fragment is covalently attached to no more than 2 nonproteinaceous polymer molecules.

13. The conjugate of claim 12 wherein the antibody fragment is attached to no more than 1 nonproteinaceous polymer molecule.

14. The conjugate of claim 8 wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH.

15. The conjugate of claim 14 wherein the antibody fragment is covalently attached to no more than 1 nonproteinaceous polymer molecule.

16. The conjugate of claim 1 wherein the nonproteinaceous polymer is a polyethylene glycol (PEG).

17. The conjugate of claim 16 wherein the PEG has an average molecular weight of at least 20kD.

18. The conjugate of claim 17 wherein the PEG has an average molecular weight of at least 40kD.

19. The conjugate of claim 18 wherein the PEG is a single chain molecule.

20. The conjugate of claim 18 wherein the PEG is a branched chain molecule.

21. The conjugate of claim 17, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is a $F(ab')_2$ and is covalently attached to no more than 2 PEG molecules.

22. The conjugate of claim 17, wherein the conjugate contains no more than one antibody fragment, and wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH and is covalently attached to no more than one PEG molecule.

23. The conjugate of claim 1 wherein the antibody fragment has an antigen binding site that binds to human IL-8.

24. The conjugate of claim 23, wherein the conjugate contains no more than one antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab' and Fab'-SH, wherein the antibody fragment is covalently attached to no more than one nonproteinaceous polymer molecule, and wherein the nonproteinaceous polymer molecule is a polyethylene glycol having an actual molecular weight of at least 30 kD.

25. The conjugate of claim I wherein the antibody fragment is humanized.

26. The conjugate of claim I wherein the conjugate contains no more than one antibody fragment.

27. A conjugate formed by one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, said antibody fragments comprising the antigen binding site or variable region of the intact antibody but free of the constant heavy chain domains of the Fc region of the intact antibody, wherein the antibody fragment comprises a heavy chain and a light chain derived from a parental antibody, wherein in the parental antibody the heavy and light chains are covalently linked by a disulfide bond between a cysteine residue in the light chain and a cysteine residue in the heavy chain, wherein in the antibody fragment the cysteine residue in the light or heavy chain is substituted with another amino acid and the cysteine residue in the opposite chain is covalently linked to a nonproteinaceous polymer molecule, wherein the apparent size of the conjugate is at least 500 kD, and wherein the molecular structure of the conjugate is free of other matter.

28. A conjugate formed by one or more antibody fragments covalently attached to one or more nonproteinaceous polymer molecules, said antibody fragments comprising the antigen binding site or variable region of the intact antibody but free of the constant heavy chain domains of the Fc region of the intact antibody, wherein the antibody fragment comprises a heavy chain and a light chain derived from a parental antibody, wherein in the parental antibody the heavy and light chains are covalently linked by a disulfide bond between a cysteine residue in the light chain and a cysteine residue in the heavy chain, wherein in the antibody fragment the cysteine residue in the light or heavy chain is substituted with another amino acid and the cysteine residue in the opposite chain is covalently linked to a nonproteinaceous polymer molecule, wherein the apparent size of the conjugate is at least 500 kD, wherein the antibody fragment incorporates a nonproteinaceous label free of any polymer, and wherein the molecular structure of the conjugate is free of other matter.

29. The conjugate of claim 28 wherein the nonproteinaceous label is a radiolabel.

30. A composition comprising the conjugate of claim 1 and a carrier.

31. The composition of claim 29 that is sterile.

32. A conjugate according to any one of claims 1 to 29, or a composition according to claim 30 or 31, for use in a method of medical treatment.

33. The use of a conjugate according to claim 23 or 24 in the preparation of a medicament for the treatment of an inflammatory disorder.

34. Use according to claim 33 wherein the inflammatory disorder is adult respiratory distress syndrome, hypovolemic shock, ulcerative colitis or rheumatoid arthritis.

Patentansprüche

1. Konjugat, das im Wesentlichen aus einem oder mehreren Antikörperfragmenten besteht, das bzw. die kovalent an ein oder mehrere nicht-proteinartige Polymermoleküle gebunden ist bzw. sind, wobei die Antikörperfragmente die Antigen-Bindungsstelle oder variable Region des intakten Antikörpers umfassen, aber frei von den konstanten Schwerketten-Domänen der Fc-Region des intakten Antikörpers sind, worin das Antikörperfragment eine Schwerkette und eine Leichtkette umfasst, die von einem elterlichen Antikörper stammen, worin im elterlichen Antikörper die Schwer- und die Leichtketten durch eine Disulfid-Bindung zwischen einem Cysteinrest in der Leichtkette und einen Cysteinrest in der Schwerkette kovalent verbunden sind, worin im Antikörperfragment der Cysteinrest in der Leicht- oder der Schwerkette durch eine andere Aminosäure substituiert ist und der Cysteinrest in der gegenüberliegenden Kette kovalent an ein nicht-proteinartiges Polymermolekül gebunden ist und worin die scheinbare Größe des Konjugats zumindest etwa 500 kD beträgt.

2. Konjugat nach Anspruch 1, worin die scheinbare Größe des Konjugats zumindest etwa 800 kD beträgt.

3. Konjugat nach Anspruch 1, worin die scheinbare Größe des Konjugats zumindest etwa 1.400 kD beträgt.

4. Konjugat nach Anspruch 1, worin die scheinbare Größe des Konjugats zumindest etwa 1.800 kD beträgt.

5. Konjugat nach Anspruch 1, worin die scheinbare Größe des Konjugats zumindest acht Mal größer als die scheinbare Größe des Antikörperfragments ist.

6. Konjugat nach Anspruch 5, worin die scheinbare Größe des Konjugats zumindest fünfzehn Mal größer als die scheinbare Größe des Antikörperfragments ist.

7. Konjugat nach Anspruch 6, worin die scheinbare Größe des Konjugats zumindest 25-mal größer als die scheinbare Größe des Antikörperfragments ist.

8. Konjugat nach Anspruch 1, worin das Konjugat nicht mehr als ein Antikörperfragment enthält und worin das Antikörperfragment aus der aus Fab, Fab', Fab'-SH, Fv, scFv und $F(ab')_2$ bestehenden Gruppe ausgewählt ist.

9. Konjugat nach Anspruch 8, worin das Antikörperfragment $F(ab')_2$ ist.

10. Konjugat nach Anspruch 1, worin das Antikörperfragment kovalent an nicht mehr als 10 nicht-proteinartige Polymermoleküle gebunden ist.

11. Konjugat nach Anspruch 10, worin das Antikörperfragment kovalent an nicht mehr als 5 nicht-proteinartige Polymermoleküle gebunden ist.

12. Konjugat nach Anspruch 11, worin das Antikörperfragment kovalent an nicht mehr als 2 nicht-proteinartige Polymermoleküle gebunden ist.

13. Konjugat nach Anspruch 12, worin das Antikörperfragment an nicht mehr als 1 nicht-proteinartiges Polymermolekül gebunden ist.

14. Konjugat nach Anspruch 8, worin das Antikörperfragment aus der aus Fab, Fab' und Fab'-SH bestehenden Gruppe ausgewählt ist.

15. Konjugat nach Anspruch 14, worin das Antikörperfragment kovalent an nicht mehr als 1 nicht-proteinartiges Polymermolekül gebunden ist.

16. Konjugat nach Anspruch 1, worin das nicht-proteinartige Polymer ein Polyethylenglykol (PEG) ist.

17. Konjugat nach Anspruch 16, worin das PEG ein mittleres Molekulargewicht von zumindest 20 kD aufweist.

18. Konjugat nach Anspruch 17, worin das PEG ein mittleres Molekulargewicht von zumindest 40 kD aufweist.

19. Konjugat nach Anspruch 18, worin das PEG ein Einzelketten-Molekül ist.

20. Konjugat nach Anspruch 18, worin das PEG ein Molekül mit verzweigter Kette ist.

21. Konjugat nach Anspruch 17, worin das Konjugat nicht mehr als ein Antikörperfragment enthält und worin das Antikörperfragment $F(ab')_2$ ist und kovalent an nicht mehr als 2 PEG-Moleküle gebunden ist.

22. Konjugat nach Anspruch 17, worin das Konjugat nicht mehr als ein Antikörperfragment enthält und worin das Antikörperfragment aus der aus Fab, Fab' und Fab'-SH bestehenden Gruppe ausgewählt ist und kovalent an nicht mehr als ein PEG-Molekül gebunden ist.

23. Konjugat nach Anspruch 1, worin das Antikörperfragment eine Antigen-Bindungsstelle aufweist, die an Human-IL-8 bindet.

24. Konjugat nach Anspruch 23, worin das Konjugat nicht mehr als ein Antikörperfragment enthält, worin das Antikörperfragment aus der aus Fab, Fab' und Fab'-SH bestehenden Gruppe ausgewählt ist, worin das Antikörperfragment kovalent an nicht mehr als ein nicht-proteinartiges Polymermolekül gebunden ist und worin das nicht-proteinartige Polymermolekül ein Polyethylenglykol mit einem tatsächlichen Molekulargewicht von zumindest 30 kD ist.

25. Konjugat nach Anspruch 1, worin das Antikörperfragment humanisiert ist.

26. Konjugat nach Anspruch 1, worin das Konjugat nicht mehr als 1 Antikörperfragment enthält.

27. Konjugat, das aus einem oder mehreren Antikörperfragmenten gebildet ist, das bzw. die kovalent an ein oder mehrere nicht-proteinartige Polymermoleküle gebunden ist bzw. sind, wobei die Antikörperfragmente die Antigen-Bindungssteile oder variable Region des intakten Antikörpers umfassen, aber frei von den konstanten Schwerkettendomänen der Fc-Region des intakten Antikörpers sind, worin das Antikörperfragment eine Schwerkette und eine Leichtkette umfasst, die von einem elterlichen Antikörper stammen, worin im elterlichen Antikörper die Schwer- und die Leichtketten durch eine Disulfidbindung zwischen einem Cysteinrest in der Leichtkette und einem Cysteinrest in der Schwerkette kovalent verbunden sind, worin im Antikörperfragment der Cysteinrest in der Leicht- oder der Schwerkette durch eine andere Aminosäure substituiert ist und der Cysteinrest in der gegenüberliegenden Kette kovalent an ein nicht-proteinartiges Polymermolekül gebunden ist, worin die scheinbare Größe des Konjugats zumindest 500 kD beträgt und worin die Molekülstruktur des Konjugats frei von anderen Substanzen ist.

28. Konjugat, das aus einem oder mehreren Antikörperfragmenten gebildet ist, das bzw. die kovalent an ein oder mehrere nicht-proteinartige Polymermoleküle gebunden ist bzw. sind, wobei die Antikörperfragmente die Antigen-Bindungsstelle oder variable Region des intakten Antikörpers umfassen, aber frei von den konstanten Schwerkettendomänen der Fc-Region des intakten Antikörpers sind, worin das Antikörperfragment eine Schwerkette und eine Leichtkette umfasst, die von einem elterlichen Antikörper stammen, worin im elterlichen Antikörper die Schwer- und die Leichtketten durch eine Disulfidbindung zwischen einem Cysteinrest in der Leichtkette und einem Cysteinrest in der Schwerkette kovalent verbunden sind, worin im Antikörperfragment der Cysteinrest in der Leicht- oder Schwerkette durch eine andere Aminosäure substituiert ist und der Cysteinrest in der gegenüberliegenden Kette kovalent an ein nicht-proteinartiges Polymermolekül gebunden ist, worin die scheinbare Größe des Konjugats zumindest 500 kD beträgt, worin das Antikörperfragment eine nicht-proteinartige Markierung umfasst, die frei von Polymer ist, und worin die Molekülstruktur des Konjugats frei von anderen Substanzen ist.

29. Konjugat nach Anspruch 28, worin die nicht-proteinartige Markierung eine Radiomarkierung ist.

30. Zusammensetzung, die das Konjugat nach Anspruch 1 und einen Träger umfasst.

31. Zusammensetzung nach Anspruch 29, die steril ist.

32. Konjugat nach einem der Ansprüche 1 bis 29 oder eine Zusammensetzung nach Anspruch 30 oder 31 zur Verwendung bei einem Verfahren zur medizinischen Behandlung.

33. Verwendung eines Konjugats nach Anspruch 23 oder 24 bei der Herstellung eines Medikaments zur Behandlung einer Entzündungserkrankung.

34. Verwendung nach Anspruch 33, worin die Entzündungserkrankung Atemnotsyndrom beim Erwachsenen, hypo-

volämischer Schock, ulzeröse Kolitis oder rheumatische Arthritis ist.

Revendications

1. Conjugué consistant essentiellement en un ou plusieurs fragments d'anticorps attachés de manière covalente à une ou plusieurs molécules de polymère non protéique, lesdits fragments d'anticorps comprenant le site de liaison de l'antigène ou la région variable de l'anticorps intact mais sans les domaines constants chaîne lourde de la région Fc de l'anticorps intact, où le fragment d'anticorps comprend une chaîne lourde et une chaîne légère dérivées d'un anticorps parent, où dans l'anticorps parent, les chaînes lourde et légère sont enchaînées de manière covalente par une liaison disulfure entre un résidu de cystéine dans la chaîne légère et un résidu de cystéine dans la chaîne lourde, où, dans le fragment d'anticorps, le résidu de cystéine dans la chaîne lourde ou légère est substitué par un autre acide aminé et le résidu de cystéine dans la chaîne opposée est enchaîné de manière covalente à une molécule de polymère non protéique, et où la dimension apparente du conjugué est d'au moins environ 500 kD.

2. Conjugué de la revendication 1, où la dimension apparente du conjugué est d'au moins environ 800 kD.

3. Conjugué de la revendication 1, où la dimension apparente du conjugué est d'au moins environ 1 400 kD.

4. Conjugué de la revendication 1, où la dimension apparente du conjugué est d'au moins environ 1 800 kD.

5. Conjugué de la revendication 1, où la dimension apparente du conjugué est au moins 8 fois plus grande que la dimension apparente du fragment d'anticorps.

6. Conjugué de la revendication 1, où la dimension apparente du conjugué est au moins 15 fois plus grande que la dimension apparente du fragment d'anticorps.

7. Conjugué de la revendication 6, où la dimension apparente du conjugué est au moins 25 fois plus grande que la dimension apparente du fragment d'anticorps.

8. Conjugué de la revendication 1, où le conjugué ne contient pas plus d'un fragment d'anticorps et où le fragment d'anticorps est sélectionné dans le groupe consistant en Fab, Fab', Fab'-SH, Fv, scFv, et $F(ab')_2$.

9. Conjugué de la revendication 8, où le fragment d'anticorps est $F(ab')_2$.

10. Conjugué de la revendication 1, où le fragment d'anticorps est attaché de manière covalente à pas plus de 10 molécules de polymère non protéique.

11. Conjugué de la revendication 10, où le fragment d'anticorps est attaché de manière covalente à pas plus de 5 molécules de polymère non protéique.

12. Conjugué de la revendication 11, où le fragment d'anticorps est attaché de manière covalente à pas plus de 2 molécules de polymère non protéique.

13. Conjugué de la revendication 12, où le fragment d'anticorps est attaché à pas plus d'1 molécule de polymère non protéique.

14. Conjugué de la revendication 8, où le fragment d'anticorps est sélectionné dans le groupe consistant en Fab, Fab' et Fab'-SH.

15. Conjugué de la revendication 14, où le fragment d'anticorps est attaché de manière covalente à pas plus d'1 molécule de polymère non protéique.

16. Conjugué de la revendication 1, où le polymère non protéique est un polyéthylène glycol (PEG).

17. Conjugué de la revendication 16, où le PEG a un poids moléculaire moyen d'au moins 20 kD.

18. Conjugué de la revendication 17, où le PEG a un poids moléculaire moyen d'au moins 40 kD.

19. Conjugué de la revendication 18, où le PEG est une molécule monocaténaire.

20. Conjugué de la revendication 18, où le PEG est une molécule à chaîne ramifiée .

21. Conjugué de la revendication 17, où le conjugué ne contient pas plus d'un fragment d'anticorps et où le fragment d'anticorps est un $F(ab')_2$ et est attaché de manière covalente à pas plus de 2 molécules de PEG.

22. Conjugué de la revendication 17, où le conjugué ne contient pas plus d'un fragment d'anticorps et où le fragment d'anticorps est sélectionné dans le groupe consistant en Fab, Fab' et Fab'-SH et est attaché de manière covalente à pas plus d'une molécule de PEG.

23. Conjugué de la revendication 1, où le fragment d'anticorps a un site de liaison d'antigène qui se lie à IL-8 humaine.

24. Conjugué de la revendication 23, où le conjugué ne contient pas plus d'un fragment d'anticorps, où le fragment d'anticorps est sélectionné dans le groupe consistant en Fab, Fab' et Fab'-SH, où le fragment d'anticorps est attaché de manière covalente à pas plus d'une molécule de polymère non protéique, et où la molécule de polymère non protéique est un polyéthylène glycol ayant un poids moléculaire réel d'au moins 30 kD.

25. Conjugué de la revendication 1, où le fragment d'anticorps est humanisé.

26. Conjugué de la revendication 1, où le conjugué ne contient pas plus d'un fragment d'anticorps.

27. Conjugué formé par un ou plusieurs fragments d'anticorps attachés de manière covalente à une ou plusieurs molécules de polymère non protéique, lesdits fragments d'anticorps comprenant le site de liaison de l'antigène ou la région variable de l'anticorps intact, mais sans les domaines constants de chaîne lourde de la région Fc de l'anticorps intact, où le fragment d'anticorps comprend une chaîne lourde et une chaîne légère dérivée d'un anticorps parent, où dans l'anticorps parent, les chaînes lourde et légère sont enchaînées de manière covalente par une liaison disulfure entre un résidu de cystéine dans la chaîne légère et un résidu de cystéine dans la chaîne lourde, où dans le fragment d'anticorps, le résidu de cystéine dans la chaîne légère ou lourde, est substitué par un autre acide aminé et le résidu de cystéine dans la chaîne opposée est enchaîné de manière covalente à une molécule de polymère non protéique, où la dimension apparente du conjugué est d'au moins 500 kD, et où la structure moléculaire du conjugué est exempte d'autre matière.

28. Conjugué formé par un ou plusieurs fragments d'anticorps attachés de manière covalente à une ou plusieurs molécules de polymère non protéique, lesdits fragments d'anticorps comprenant le site de liaison de l'antigène ou la région variable de l'anticorps intact, mais sans les domaines constants de chaîne lourde de la région Fc de l'anticorps intact, où le fragment d'anticorps comprend une chaîne lourde et une chaîne légère dérivée d'un anticorps parent, où dans l'anticorps parent, les chaînes lourde et légère sont enchaînées de manière covalente par une liaison disulfure entre un résidu de cystéine dans la chaîne légère et un résidu de cystéine dans la chaîne lourde, où, dans le fragment d'anticorps, le résidu de cystéine dans la chaîne légère ou lourde est substitué par un autre acide aminé et le résidu de cystéine dans la chaîne opposée est enchaîné de manière covalente à une molécule de polymère non protéique, où la dimension apparente du conjugué est d'au moins 500 kD, où le fragment d'anticorps incorpore un marqueur non protéique exempt de tout polymère, et où la structure moléculaire du conjugué est exempte de toute autre matière.

29. Conjugué de la revendication 28, où le marqueur non protéique est un radiomarqueur.

30. Composition comprenant le conjugué de la revendication 1 et un support.

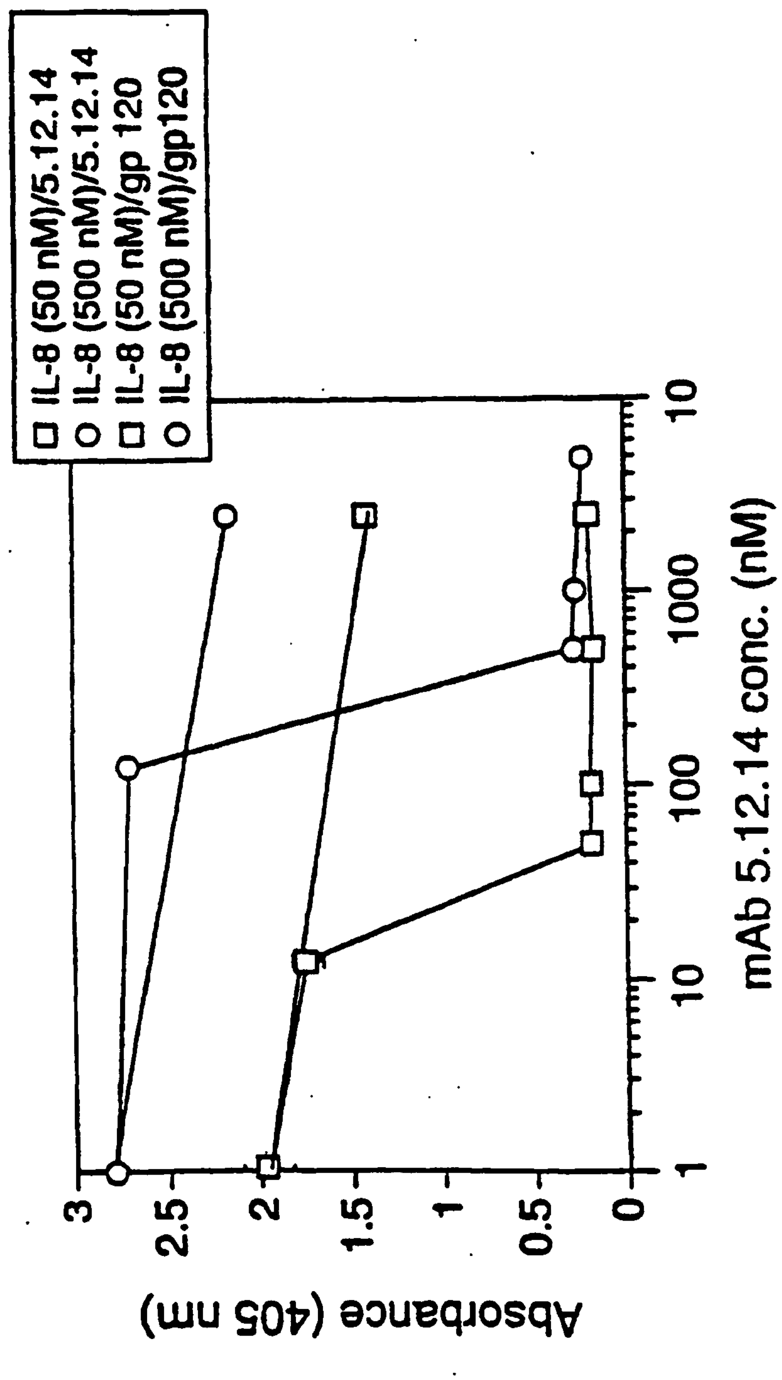
31. Composition de la revendication 29 qui est stérile.

32. Conjugué selon l'une quelconque des revendications 1 à 29, ou composition selon la revendication 30 ou 31 pour une utilisation dans une méthode de traitement médical.

33. Utilisation d'un conjugué selon la revendication 23 ou 24 dans la préparation d'un médicament pour le traitement d'un trouble inflammatoire.

34. Utilisation selon la revendication 33 où le trouble inflammatoire est un syndrome de détresse respiratoire chez l'adulte, un choc hypovolémique, une colite ulcérative ou une arthrite rhumatoïde.

FIG. 1



IL-8 (50 nM)/5.12.14
IL-8 (500 nM)/5.12.14
IL-8 (50 nM)/gp 120
IL-8 (500 nM)/gp120
Absorbance (405 nm)
0
0.5
1
1.5
2
2.5
3
1
10
100
1000
10
mAb 5.12.14 conc. (nM)

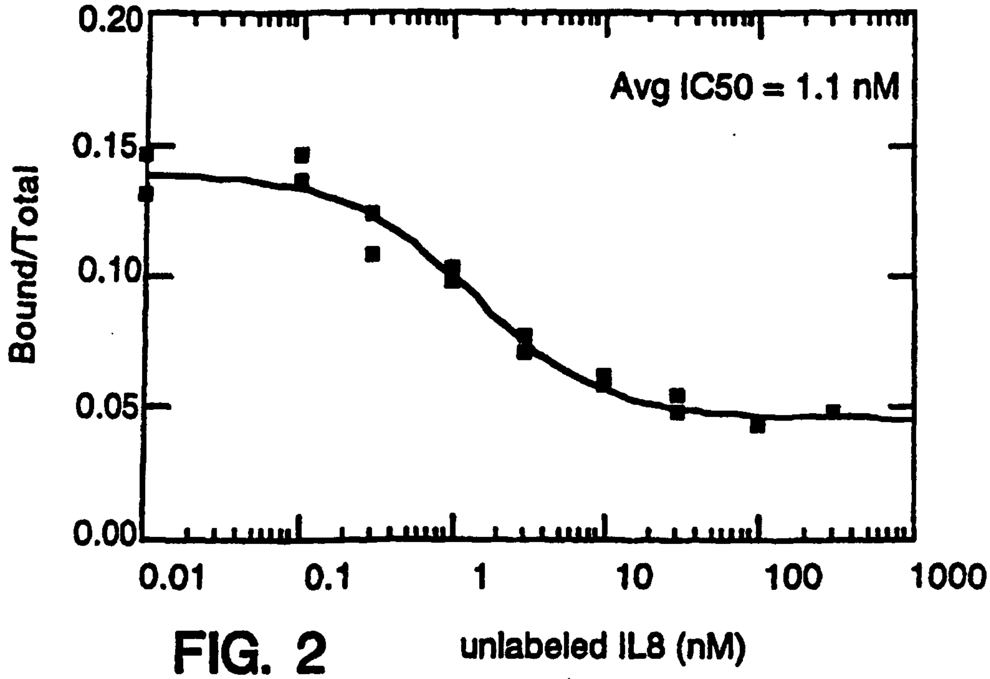

0.20
0.15
0.10
0.05
0.00
Bound/Total
Avg IC50 = 1.1 nM
0.01
0.1
1
10
100
1000
unlabeled IL8 (nM)

FIG. 2

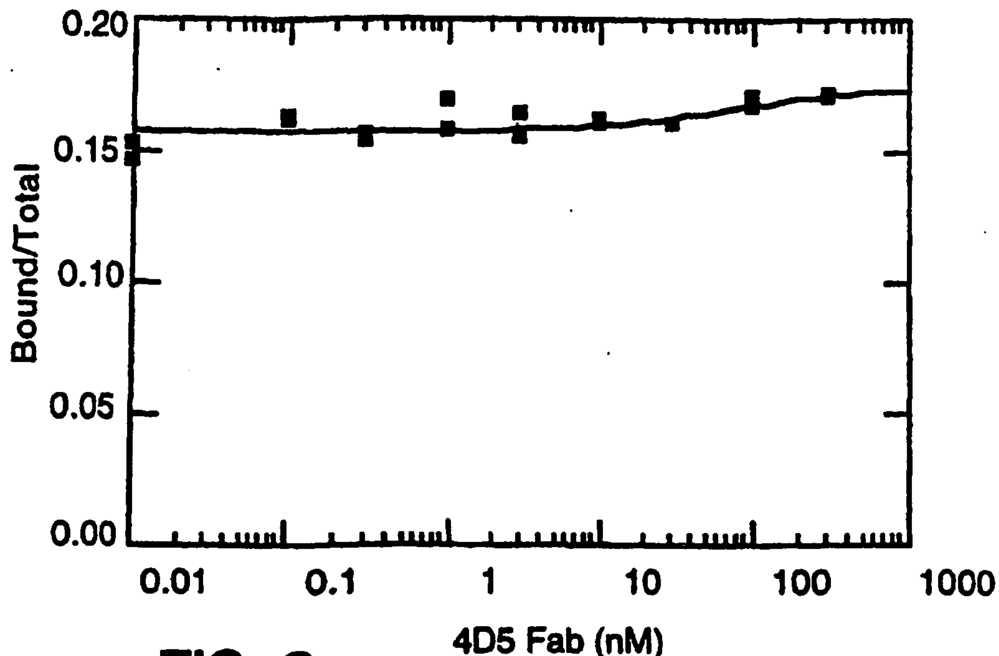

0.20
0.15
0.10
0.05
0.00
Bound/Total
0.01
0.1
1
10
100
1000
4D5 Fab (nM)

FIG. 3

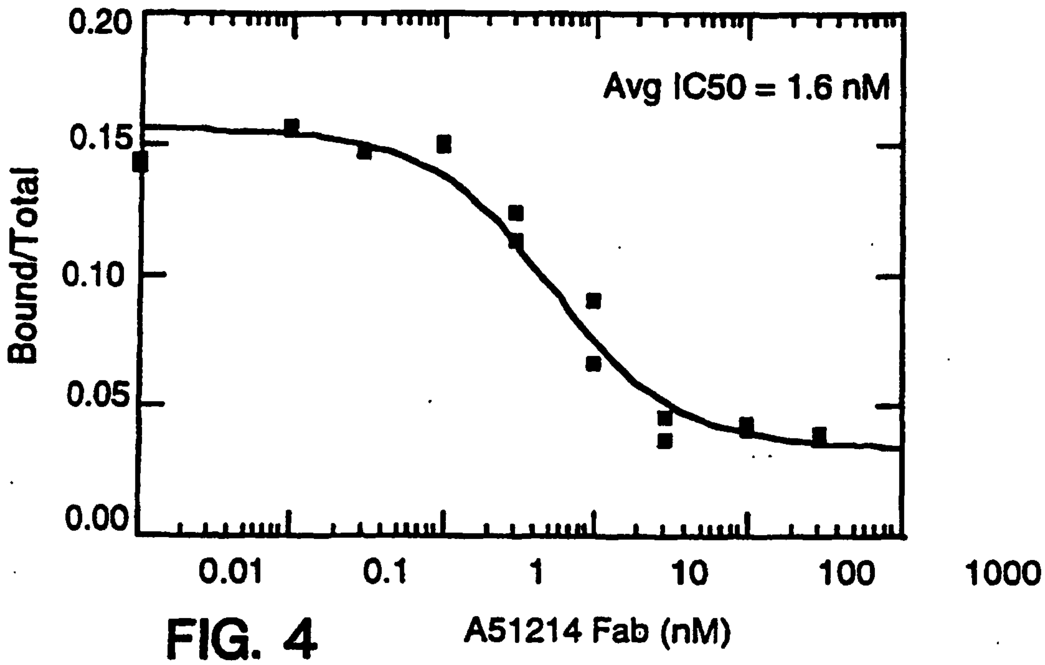

0.20
0.15
0.10
0.05
0.00
Bound/Total
Avg IC50 = 1.6 nM
0.01
0.1
1
10
100
1000
A51214 Fab (nM)

FIG. 4

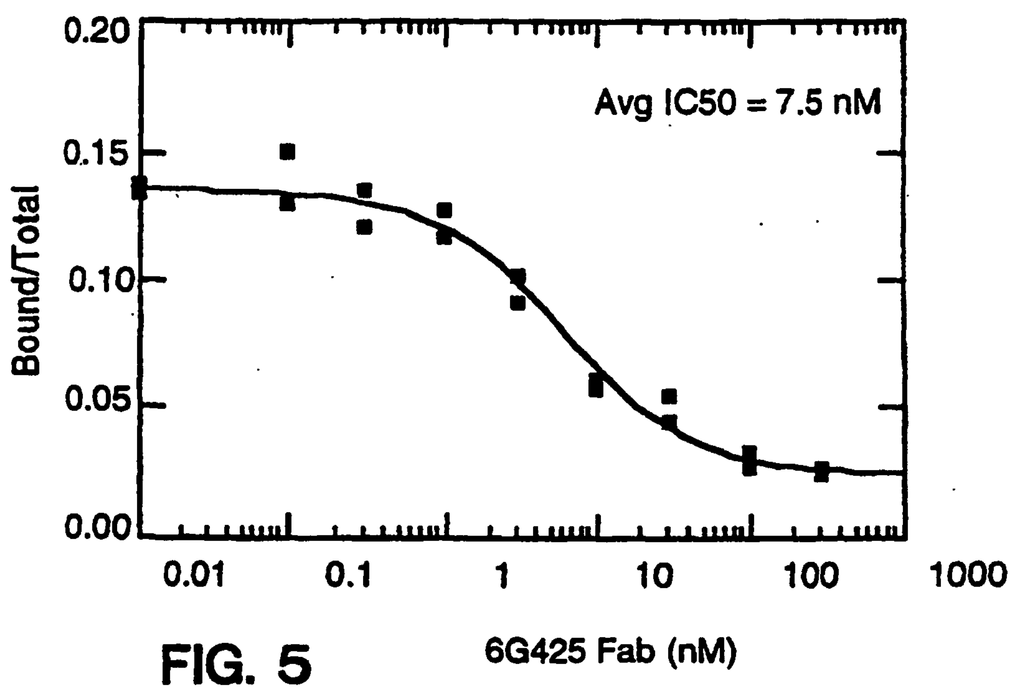
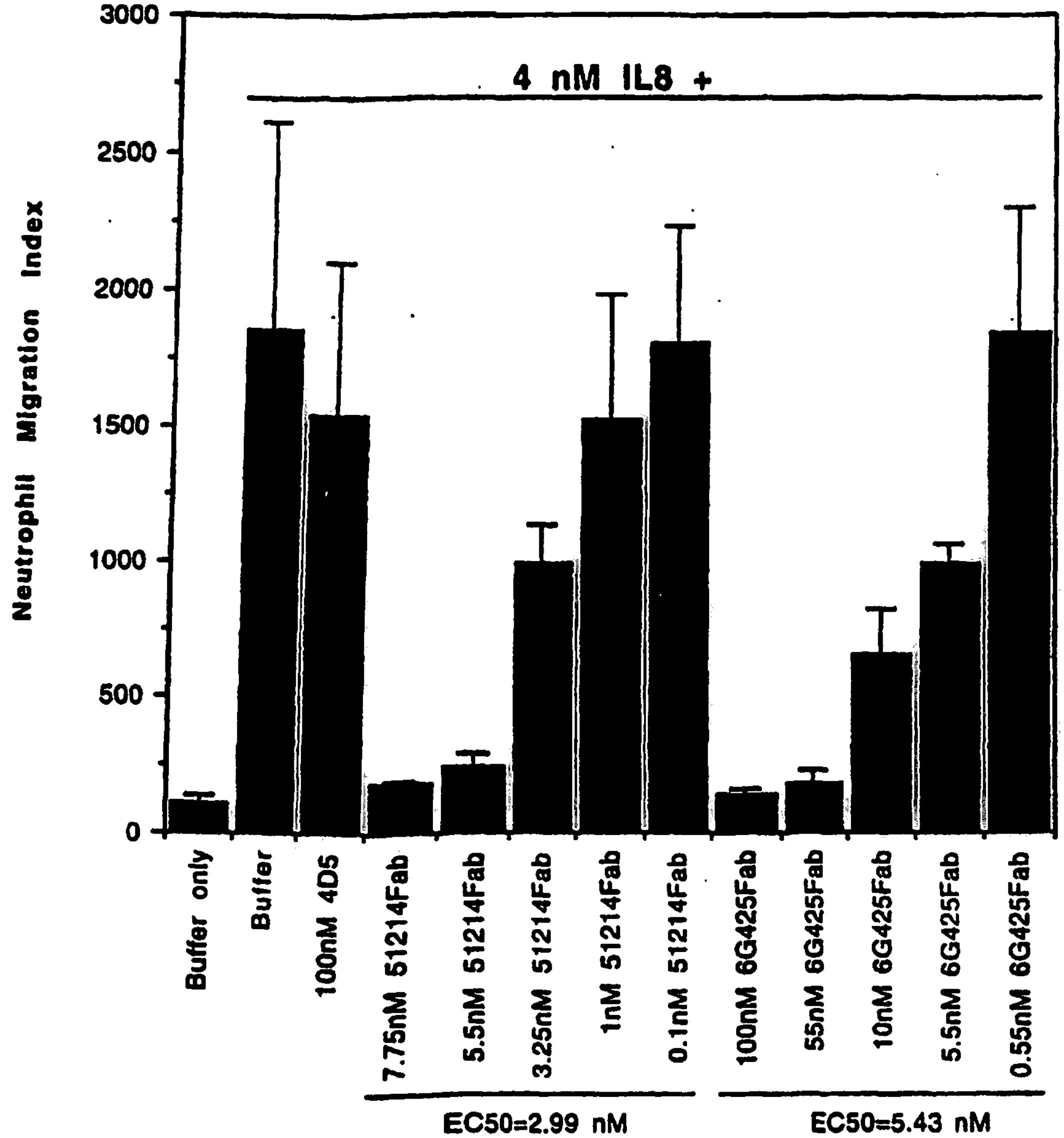

0.20
0.15
0.10
0.05
0.00
Bound/Total
Avg IC50 = 7.5 nM
0.01
0.1
1
10
100
1000
6G425 Fab (nM)

FIG. 5



Neutrophil Migration Index
3000
2500
2000
1500
1000
500
0
4 nM IL8 +
Buffer only
Buffer
100nM 4D5
7.75nM 51214Fab
5.5nM 51214Fab
3.25nM 51214Fab
1nM 51214Fab
0.1nM 51214Fab
100nM 6G425Fab
55nM 6G425Fab
10nM 6G425Fab
5.5nM 6G425Fab
0.55nM 6G425Fab
EC50=2.99 nM
EC50=5.43 nM

FIG. 6

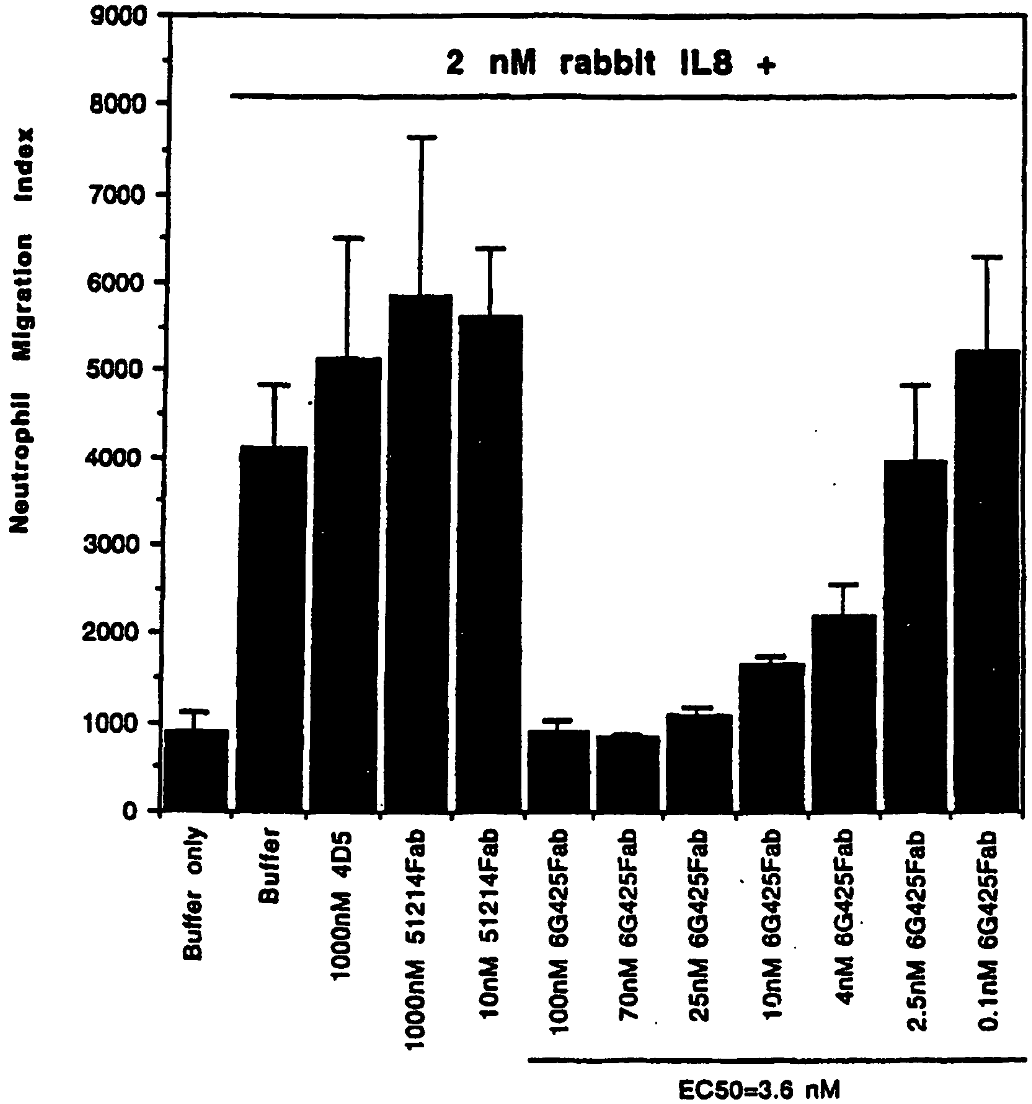

2 nM rabbit IL8 +
Neutrophil Migration Index
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Buffer only
Buffer
1000nM 4D5
1000nM 51214Fab
10nM 51214Fab
100nM 6G425Fab
70nM 6G425Fab
25nM 6G425Fab
10nM 6G425Fab
4nM 6G425Fab
2.5nM 6G425Fab
0.1nM 6G425Fab
EC50=3.6 nM

FIG. 7

FIG. 8

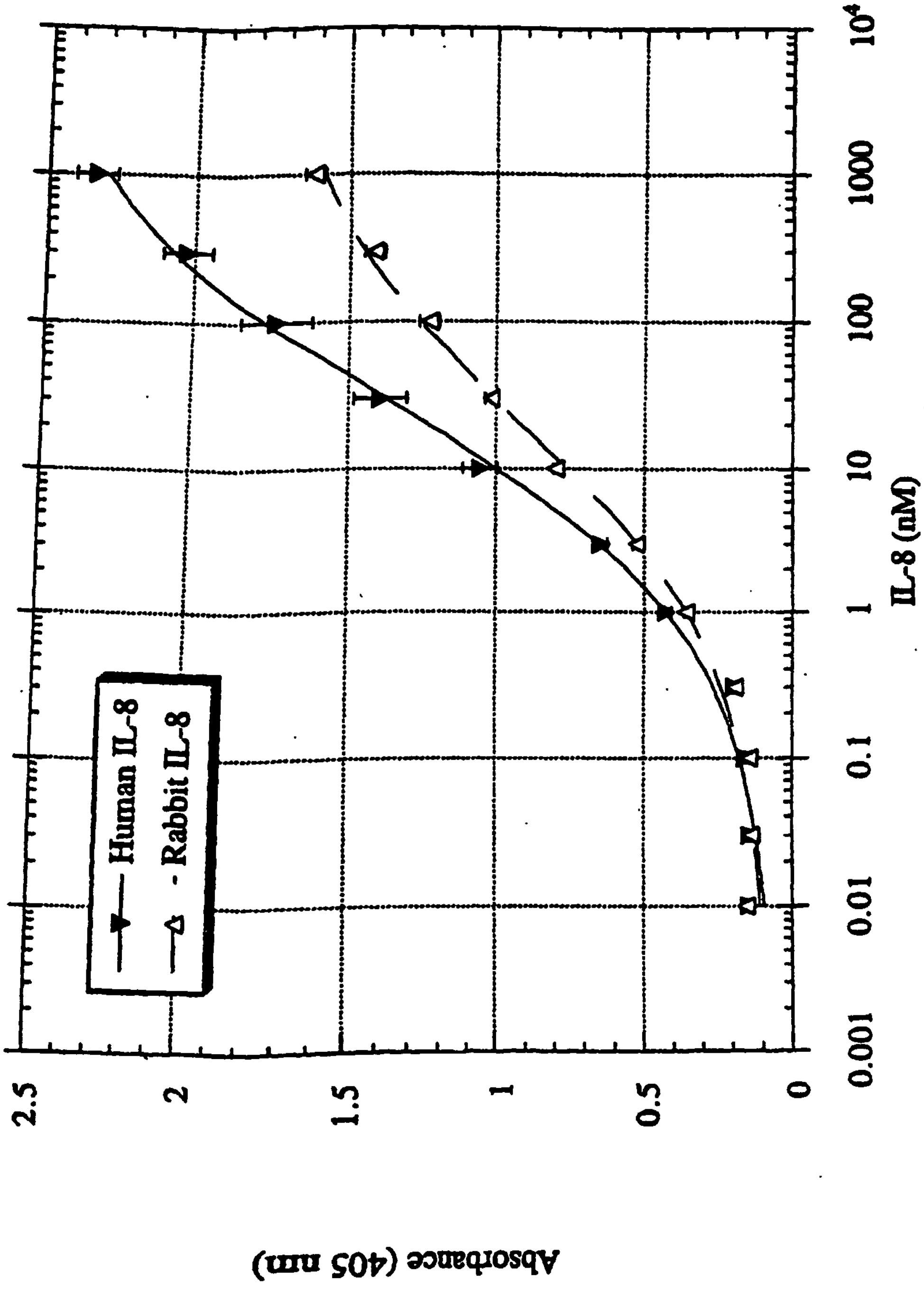

Human IL-8
Rabbit IL-8
Absorbance (405 nm)
IL-8 (nM)
0
0.5
1
1.5
2
2.5
0.001
0.01
0.1
1
10
100
1000
10^4

FIG. 9

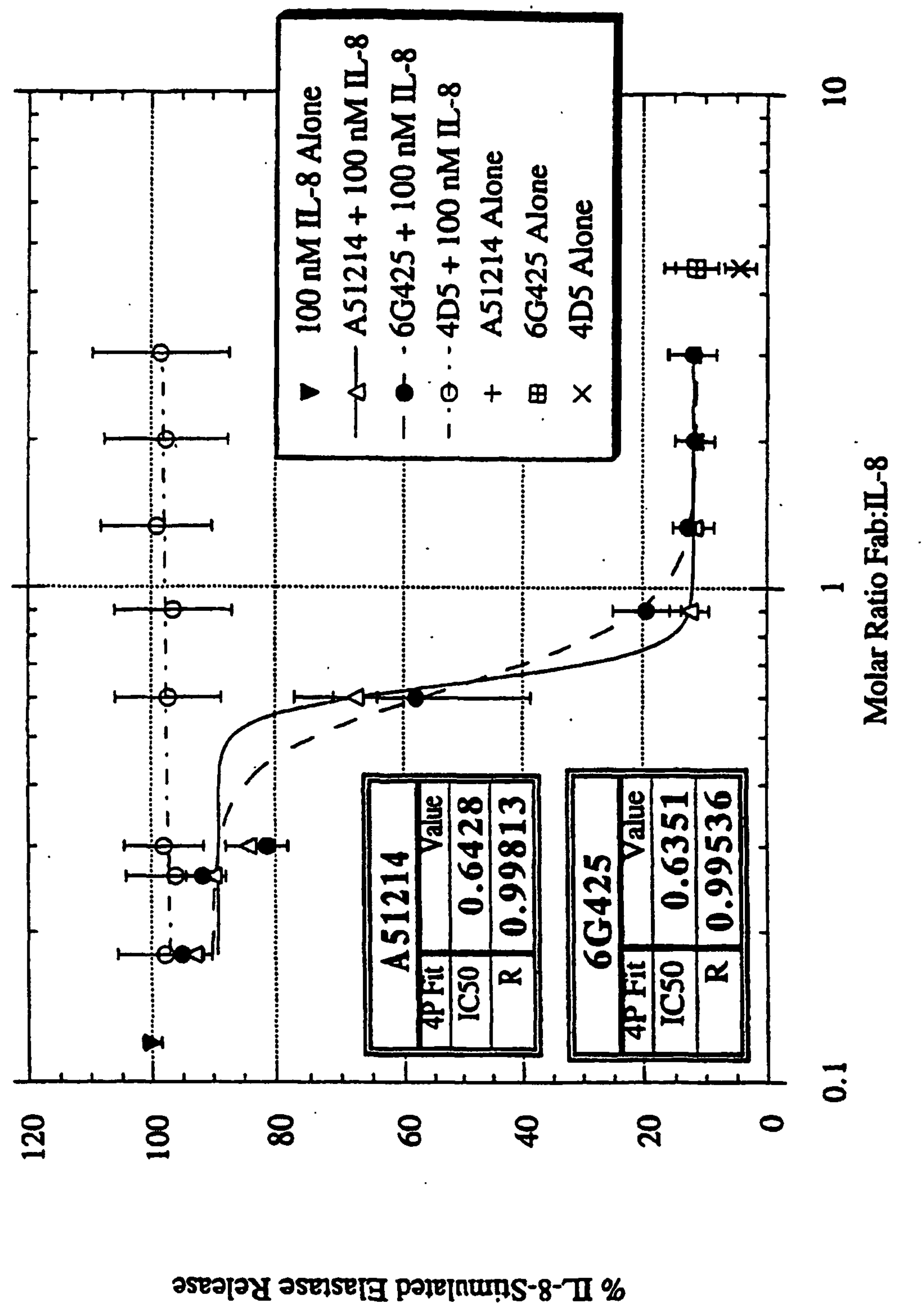
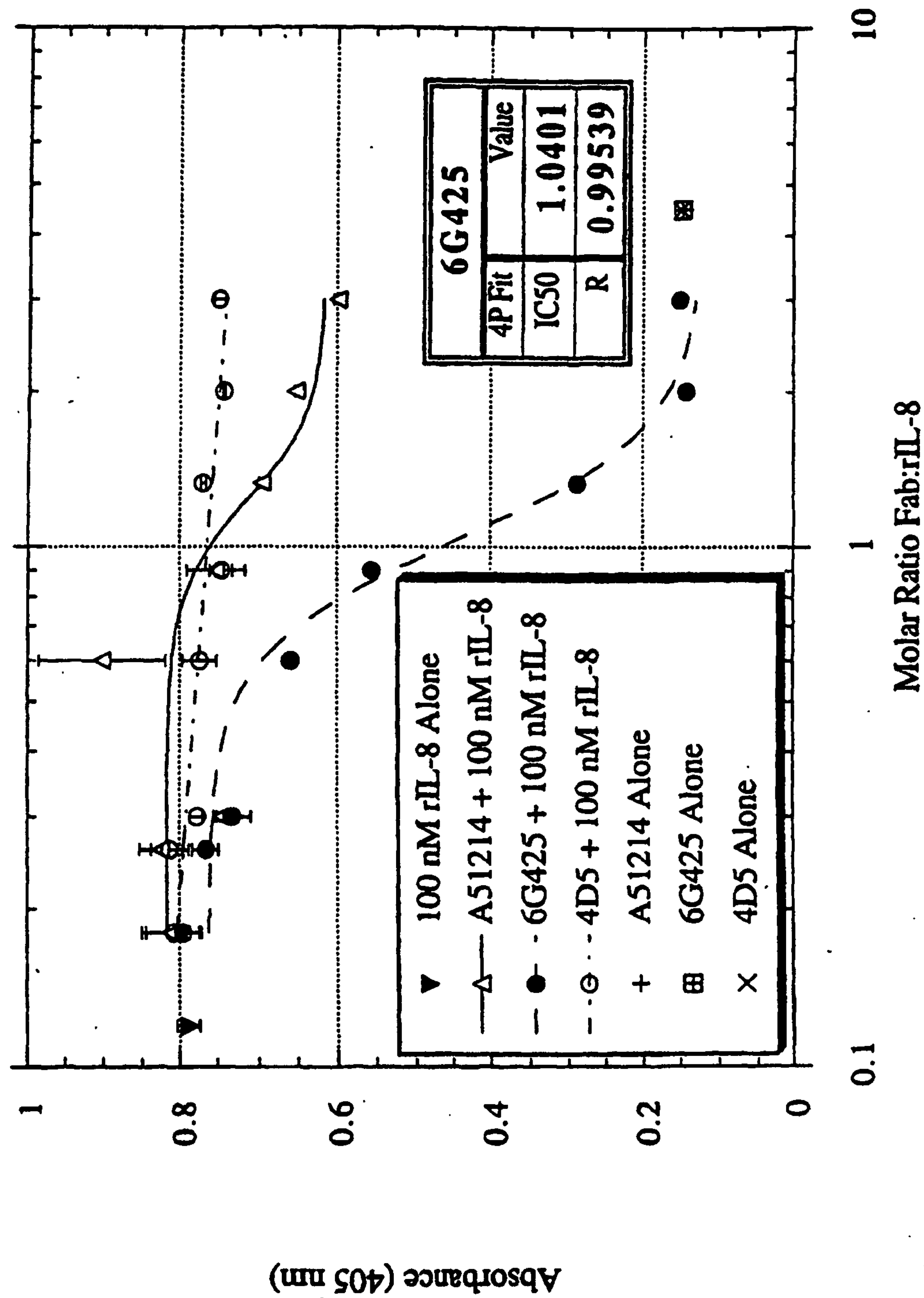

% IL-8-Stimulated Elastase Release
Molar Ratio Fab:IL-8
120
100
80
60
40
20
0
0.1
1
10
100 nM IL-8 Alone
A51214 + 100 nM IL-8
6G425 + 100 nM IL-8
4D5 + 100 nM IL-8
A51214 Alone
6G425 Alone
4D5 Alone
A51214
4P Fit
Value
IC50
0.6428
R
0.99813
6G425
4P Fit
Value
IC50
0.6351
R
0.99536

FIG. 10



Absorbance (405 nm)
Molar Ratio Fab:rIL-8
100 nM rIL-8 Alone
A51214 + 100 nM rIL-8
6G425 + 100 nM rIL-8
4D5 + 100 nM rIL-8
A51214 Alone
6G425 Alone
4D5 Alone
6G425
4P Fit
Value
IC50
1.0401
R
0.99539

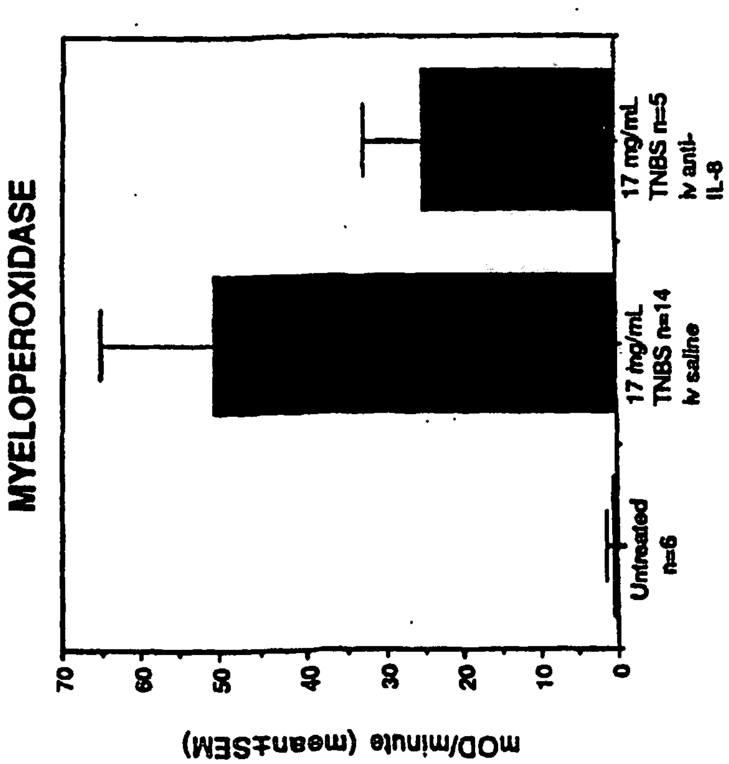


FIG. 11A

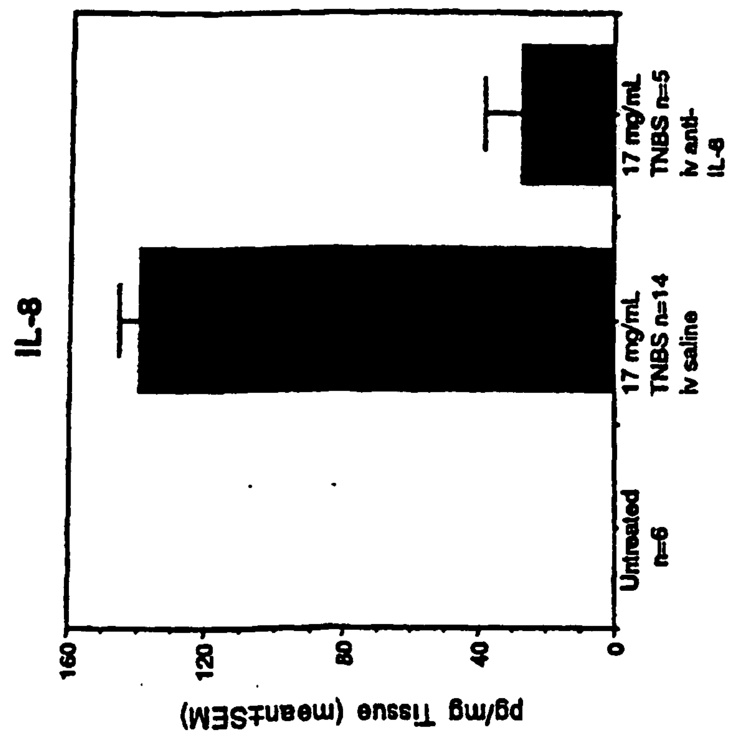


FIG. 11B

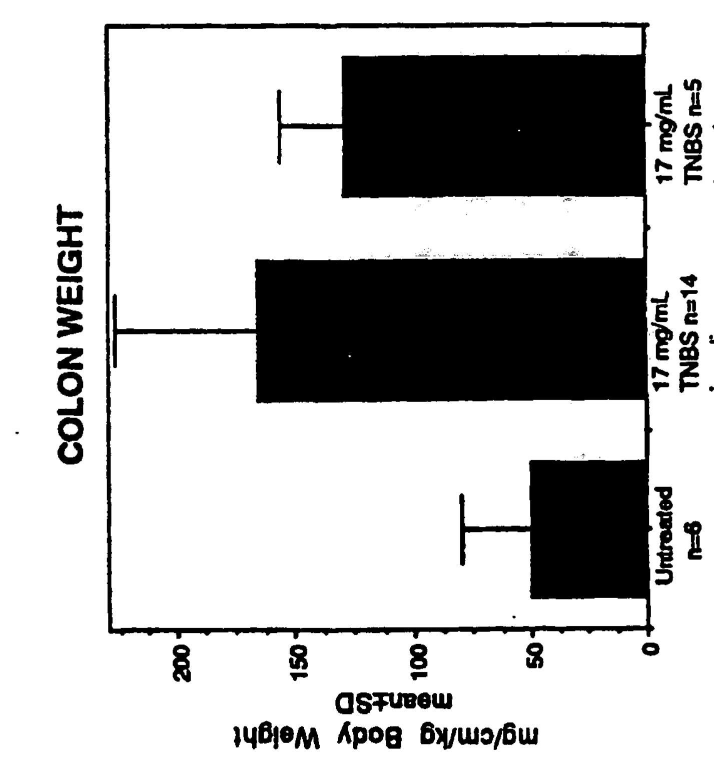


FIG. 11C

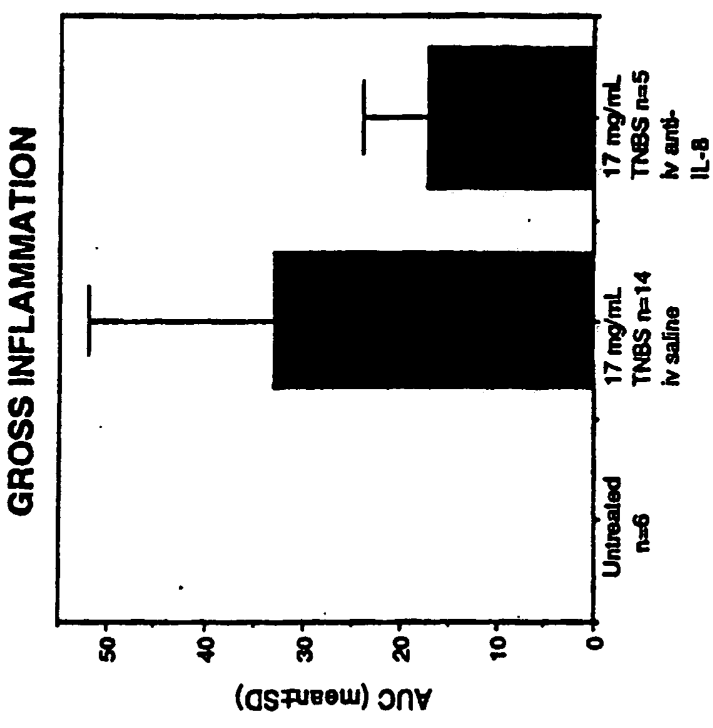


FIG. 11D

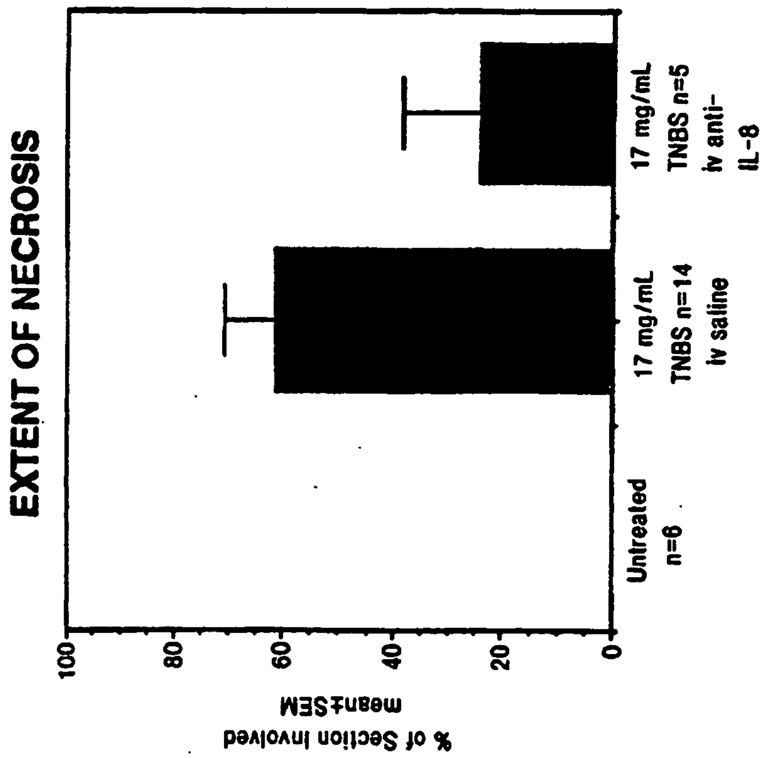


FIG. 11F

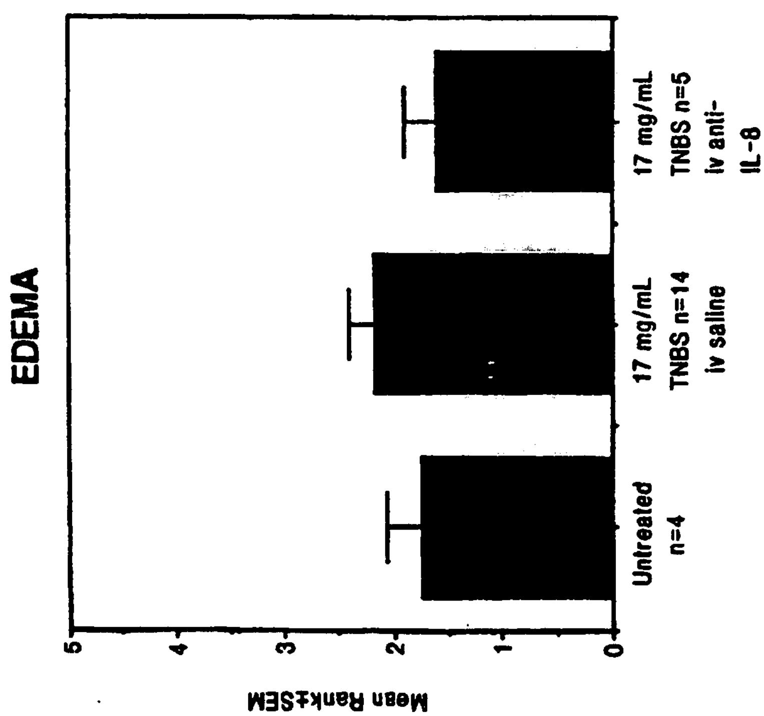


FIG. 11E

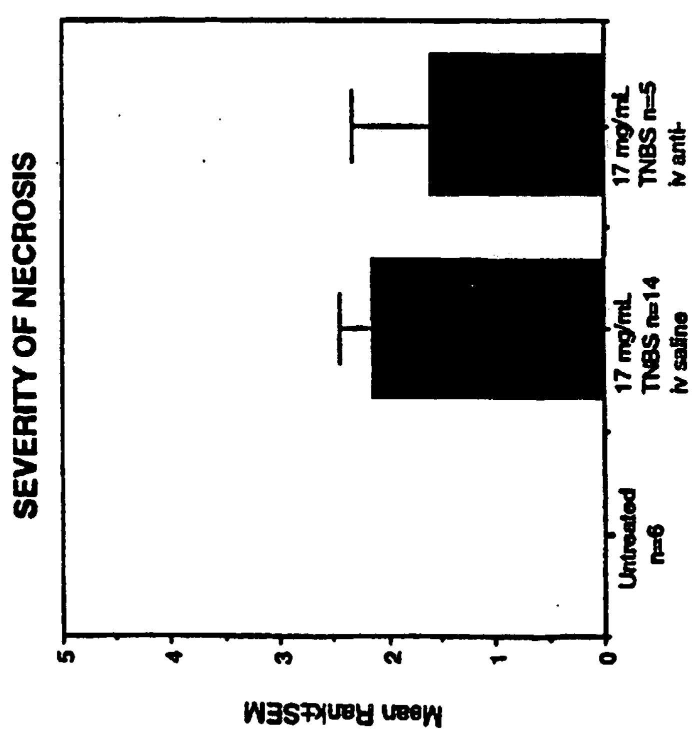


FIG. 11G

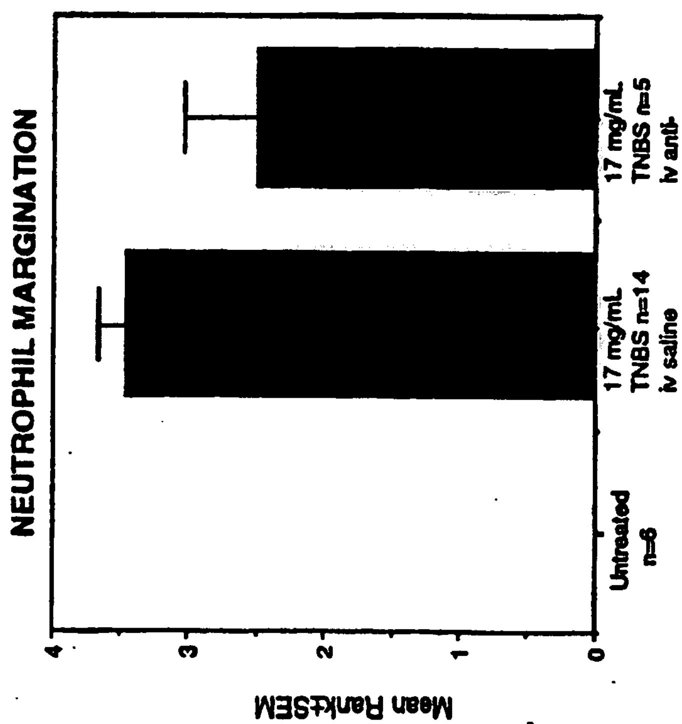


FIG. 11H

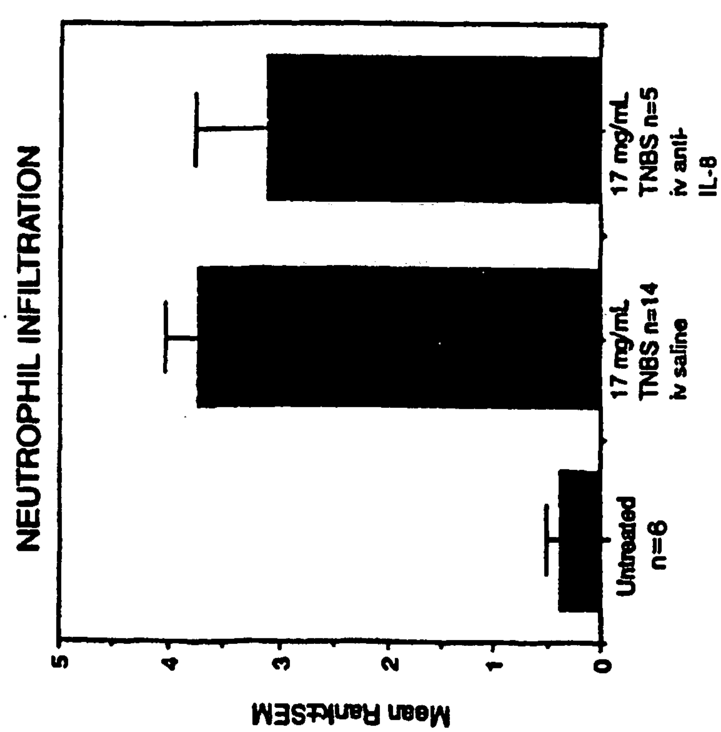


FIG. 11I

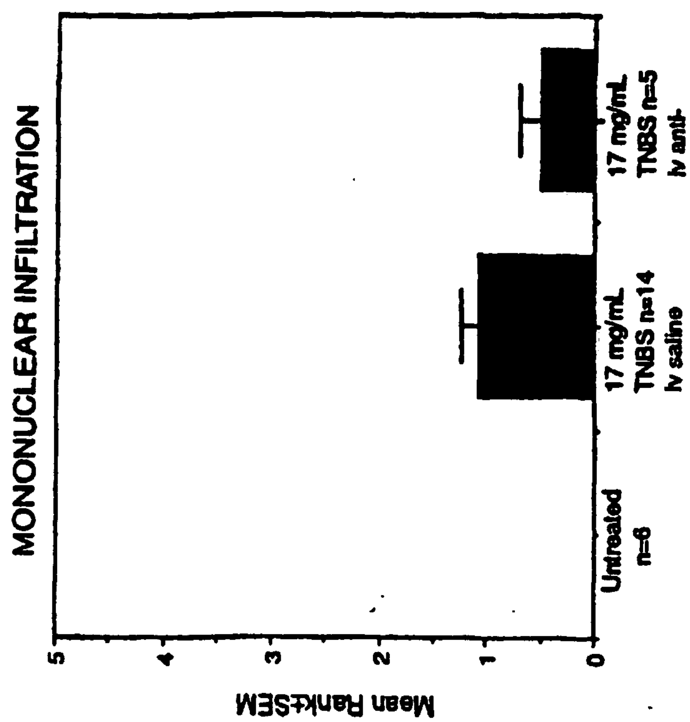
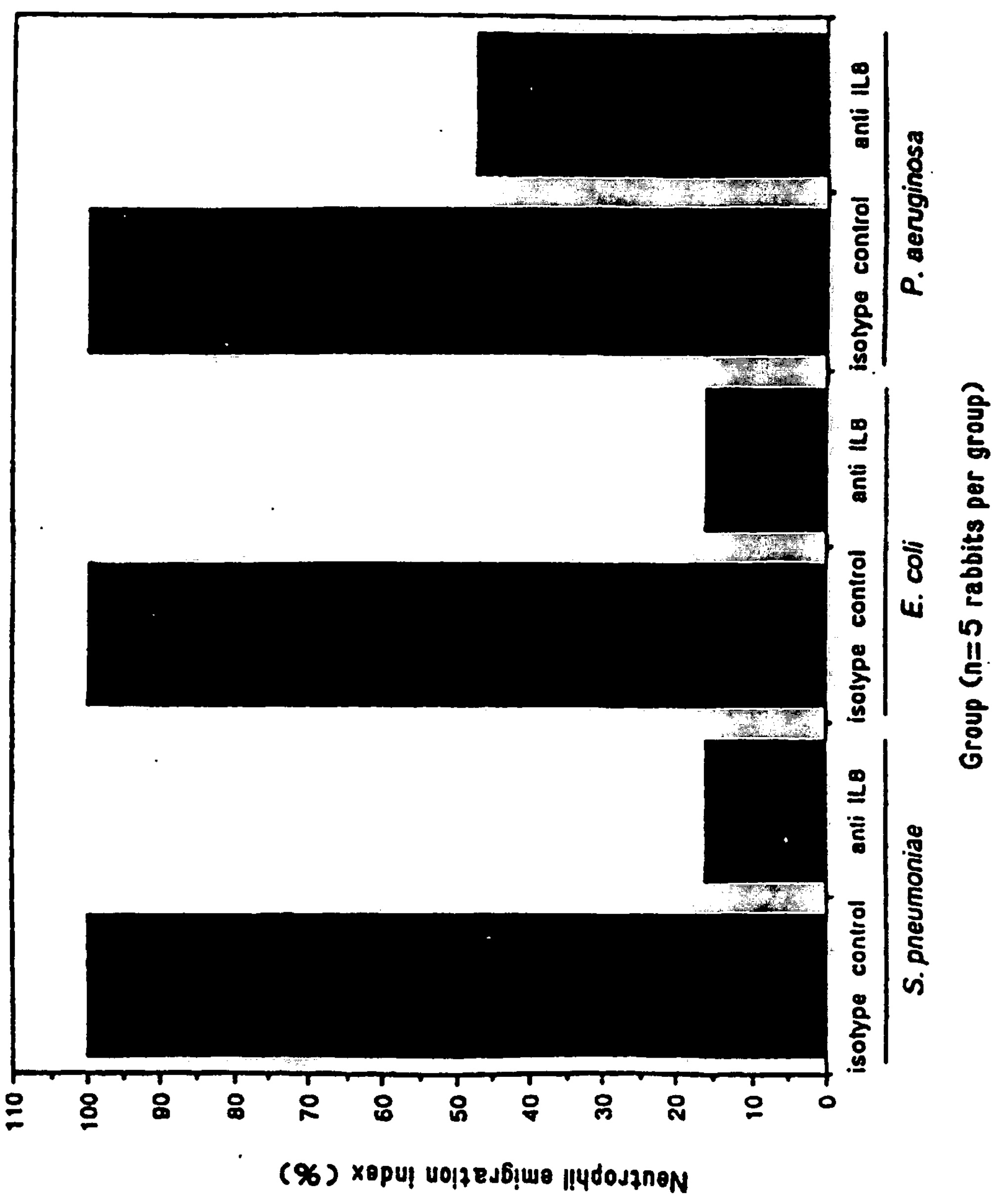


FIG. 11J

FIG. 12


Neutrophil emigration index (%)
0
10
20
30
40
50
60
70
80
90
100
110
isotype control
anti IL8
isotype control
anti IL8
isotype control
anti IL8
S. pneumoniae
E. coli
P. aeruginosa
Group (n=5 rabbits per group)

Light Chain Primers:

FIG. 13

MKLC-1, 22mer

5' CAGTCCAACTGTTCAGGACGCC 3'

MKLC-2, 22mer

5' GTGCTGCTCATGCTGTAGGTGC 3'

MKLC-3, 23mer

5' GAAGTTGATGTCTTGTGAGTGGC 3'

Heavy Chain Primers:

IGG2AC-1, 24mer

5' GCATCCTAGAGTCACCGAGGAGCC 3'

IGG2AC-2, 22mer

5' CACTGGCTCAGGGAAATAACCC 3'

IGG2AC-3, 22mer

5' GGAGAGCTGGGAAGGTGTGCAC 3'

FIG. 14

Light chain forward primer

SL001A-2 35 mer

```
5' ACAAACGCGTACGCT GACATCGTCATGACCCAGTC  3'
                     T  T        T
                                 A
```

Light chain reverse primer

SL001B 37 mer

5' GCTCTTCGAATG GTGGGAAGATGGATACAGTTGGTGC 3'

FIG. 15

Heavy chain forward primer

SL002B 39 mer

```
5' CGATGGGCCCGG ATAGACCGATGGGGCTGTTGTTTTGGC 3'
                      T           C
                      G
                      A
```

Heavy chain reverse primer

SL002B 39-MER

```
5' CGATGGGCCCGG ATAGACCGATGGGGCTGTTGTTTTGGC 3'
                      T
                      A
                      G
```

```
  1 GACATTGTCA TGACACAGTC TCAAAAATTC ATGTCCACAT CAGTAGGAGA CAGGGTCAGC
    CTGTAACAGT ACTGTGTCAG AGTTTTTAAG TACAGGTGTA GTCATCCTCT GTCCCAGTCG
  1 D  I  V  M   T  Q  S   Q  K  F   M  S  T  S   V  G  D   R  V  S

 61 GTCACCTGCA AGGCCAGTCA GAATGTGGGT ACTAATGTAG CCTGGTATCA ACAGAAACCA
    CAGTGGACGT TCCGGTCAGT CTTACACCCA TGATTACATC GGACCATAGT TGTCTTTGGT
 21 V  T  C  K   A  S  Q   N  V  G   T  N  V  A   W  Y  Q   Q  K  P
             *   *  *  *   *  *  *   *  *  *  *
                            CDR #1

121 GGGCAATCTC CTAAAGCACT GATTTACTCG TCATCCTACC GGTACAGTGG AGTCCCTGAT
    CCCGTTAGAG GATTTCGTGA CTAAATGAGC AGTAGGATGG CCATGTCACC TCAGGGACTA
 41 G  Q  S  P   K  A  L   I  Y  S   S  S  Y  R   Y  S  G   V  P  D
                                *   *  *  *  *   *  *
                                      CDR #2

181 CGCTTCACAG GCAGTGGATC TGGGACAGAT TTCACTCTCA CCATCAGCCA TGTGCAGTCT
    GCGAAGTGTC CGTCACCTAG ACCCTGTCTA AAGTGAGAGT GGTAGTCGGT ACACGTCAGA
 61 R  F  T  G   S  G  S   G  T  D   F  T  L  T   I  S  H   V  Q  S

241 GAAGACTTGG CAGACTATTT CTGTCAGCAA TATAACATCT ATCCTCTCAC GTTCGGTCCT
    CTTCTGAACC GTCTGATAAA GACAGTCGTT ATATTGTAGA TAGGAGAGTG CAAGCCAGGA
 81 E  D  L  A   D  Y  F   C  Q  Q   Y  N  I  Y   P  L  T   F  G  P
                             *  *   *  *  *  *   *  *  *
                                  CDR #3

301 GGGACCAAGC TGGAGTTGAA ACGGGCTGAT GCTGCACCAC CAACTGTATC CATCTTCCCA
    CCCTGGTTCG ACCTCAACTT TGCCCGACTA CGACGTGGTG GTTGACATAG GTAGAAGGGT
101 G  T  K  L   E  L  K   R  A  D   A  A  P  P   T  V  S   I  F  P

      BstBI
361 CCATTCGAA
    GGTAAGCTT
121 P  F  E
```

FIG. 16

```
  1 TTCTATTGCT ACAAACGCGT ACGCTGAGGT GCAGCTGGTG GAGTCTGGGG GAGGCTTAGT
    AAGATAACGA TGTTTGCGCA TGCGACTCCA CGTCGACCAC CTCAGACCCC CTCCGAATCA
  1                       E  V   Q  L  V   E  S  G  G   G  L  V

 61 GCCGCCTGGA GGGTCCCTGA AACTCTCCTG TGCAGCCTCT GGATTCATAT TCAGTAGTTA
    CGGCGGACCT CCCAGGGACT TTGAGAGGAC ACGTCGGAGA CCTAAGTATA AGTCATCAAT
 13 P  P  G   G  S  L  K   L  S  C   A  A  S   G  F  I  F   S  S  Y
                                                             *  *
                                                    CDR #1

121 TGGCATGTCT TGGGTTCGCC AGACTCCAGG CAAGAGCCTG GAGTTGGTCG CAACCATTAA
    ACCGTACAGA ACCCAAGCGG TCTGAGGTCC GTTCTCGGAC CTCAACCAGC GTTGGTAATT
 33 G  M  S   W  V  R  Q   T  P  G   K  S  L   E  L  V  A   T  I  N
    *  *  *                                                 *  *  *

181 TAATAATGGT GATAGCACCT ATTATCCAGA CAGTGTGAAG GGCCGATTCA CCATCTCCCG
    ATTATTACCA CTATCGTGGA TAATAGGTCT GTCACACTTC CCGGCTAAGT GGTAGAGGGC
 53 N  N  G   D  S  T  Y   Y  P  D   S  V  K   G  R  F  T   I  S  R
    *  *  *   *  *  *  *   *  *  *   *  *  *
                 CDR #2

241 AGACAATGCC AAGAACACCC TGTACCTGCA AATGAGCAGT CTGAAGTCTG AGGACACAGC
    TCTGTTACGG TTCTTGTGGG ACATGGACGT TTACTCGTCA GACTTCAGAC TCCTGTGTCG
 73 D  N  A   K  N  T  L   Y  L  Q   M  S  S   L  K  S  E   D  T  A

301 CATGTTTTAC TGTGCAAGAG CCCTCATTAG TTCGGCTACT TGGTTTGGTT ACTGGGGCCA
    GTACAAAATG ACACGTTCTC GGGAGTAATC AAGCCGATGA ACCAAACCAA TGACCCCGGT
 93 M  F  Y   C  A  R  A   L  I  S   S  A  T   W  F  G  Y   W  G  Q
                        *   *  *  *   *  *  *   *  *  *  *
                                  CDR #3

361 AGGGACTCTG GTCACTGTCT CTGCAGCCAA AACAACAGCC CCATCTGTCT
    TCCCTGAGAC CAGTGACAGA GACGTCGGTT TTGTTGTCGG GGTAGACAGA
113 G  T  L   V  T  V  S   A  A  K   T  T  A   P  S  V  Y

       ApaI
411   ATCCGGG
      TAGGCCC
130   P
```

FIG. 17

FIG. 18

VL.front 31-MER

5' ACAAACGCGTACGCTGATATCGTCATGACAG 3'

VL.rear 31-MER

5' GCAGCATCAGCTCTTCGAAGCTCCAGCTTGG 3'

VH.front.SPE 21-MER

5' CCACTAGTACGCAAGTTCACG 3'

VH.rear 33-MER

5' GATGGGCCCTTGGTGGAGGCTGCAGAGACAGTG 3'

```
  1 ATGAAGAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTCTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

 61 GCGTACGCTG ATATCGTCAT GACACAGTCT CAAAAATTCA TGTCCACATC AGTAGGAGAC
    CGCATGCGAC TATAGCAGTA CTGTGTCAGA GTTTTTAAGT ACAGGTGTAG TCATCCTCTG
 -3 A  Y  A  D   I  V  M   T  Q  S   Q  K  F  M   S  T  S   V  G  D

121 AGGGTCAGCG TCACCTGCAA GGCCAGTCAG AATGTGGGTA CTAATGTAGC CTGGTATCAA
    TCCCAGTCGC AGTGGACGTT CCGGTCAGTC TTACACCCAT GATTACATCG GACCATAGTT
 18 R  V  S  V   T  C  K   A  S  Q   N  V  G  T   N  V  A   W  Y  Q
                      *   *  *  *   *  *  *  *   *  *  *
                                    CDR #1

181 CAGAAACCAG GGCAATCTCC TAAAGCACTG ATTTACTCGT CATCCTACCG GTACAGTGGA
    GTCTTTGGTC CCGTTAGAGG ATTTCGTGAC TAAATGAGCA GTAGGATGGC CATGTCACCT
 38 Q  K  P  G   Q  S  P   K  A  L   I  Y  S  S   S  Y  R   Y  S  G
                                          *  *   *  *  *   *  *
                                                 CDR #2

241 GTCCCTGATC GCTTCACAGG CAGTGGATCT GGGACAGATT TCACTCTCAC CATCAGCCAT
    CAGGGACTAG CGAAGTGTCC GTCACCTAGA CCCTGTCTAA AGTGAGAGTG GTAGTCGGTA
 58 V  P  D  R   F  T  G   S  G  S   G  T  D  F   T  L  T   I  S  H

301 GTGCAGTCTG AAGACTTGGC AGACTATTTC TGTCAGCAAT ATAACATCTA TCCTCTCACG
    CACGTCAGAC TTCTGAACCG TCTGATAAAG ACAGTCGTTA TATTGTAGAT AGGAGAGTGC
 78 V  Q  S  E   D  L  A   D  Y  F   C  Q  Q  Y   N  I  Y   P  L  T
                                       *  *  *   *  *  *   *  *  *
                                                  CDR #3
                       BstBI
361 TTCGGTCCTG GGACCAAGCT GGAGCTTCGA AGAGCTGTGG CTGCACCATC TGTCTTCATC
    AAGCCAGGAC CCTGGTTCGA CCTCGAAGCT TCTCGACACC GACGTGGTAG ACAGAAGTAG
 98 F  G  P  G   T  K  L   E  L  R   R  A  V  A   A  P  S   V  F  I

421 TTCCCGCCAT CTGATGAGCA GTTGAAATCT GGAACTGCTT CTGTTGTGTG CCTGCTGAAT
    AAGGGCGGTA GACTACTCGT CAACTTTAGA CCTTGACGAA GACAACACAC GGACGACTTA
118 F  P  P  S   D  E  Q   L  K  S   G  T  A  S   V  V  C   L  L  N

481 AACTTCTATC CCAGAGAGGC CAAAGTACAG TGGAAGGTGG ATAACGCCCT CCAATCGGGT
    TTGAAGATAG GGTCTCTCCG GTTTCATGTC ACCTTCCACC TATTGCGGGA GGTTAGCCCA
138 N  F  Y  P   R  E  A   K  V  Q   W  K  V  D   N  A  L   Q  S  G

541 AACTCCCAGG AGAGTGTCAC AGAGCAGGAC AGCAAGGACA GCACCTACAG CCTCAGCAGC
    TTGAGGGTCC TCTCACAGTG TCTCGTCCTG TCGTTCCTGT CGTGGATGTC GGAGTCGTCG
158 N  S  Q  E   S  V  T   E  Q  D   S  K  D  S   T  Y  S   L  S  S

601 ACCCTGACGC TGAGCAAAGC AGACTACGAG AAACACAAAG TCTACGCCTG CGAAGTCACC
    TGGGACTGCG ACTCGTTTCG TCTGATGCTC TTTGTGTTTC AGATGCGGAC GCTTCAGTGG
178 T  L  T  L   S  K  A   D  Y  E   K  H  K  V   Y  A  C   E  V  T

661 CATCAGGGCC TGAGCTCGCC CGTCACAAAG AGCTTCAACA GGGGAGAGTG
    GTAGTCCCGG ACTCGAGCGG GCAGTGTTTC TCGAAGTTGT CCCCTCTCAC
198 H  Q  G  L   S  S  P   V  T  K   S  F  N  R   G  E  C

711   TTAA
      AATT
```

FIG. 19

```
  1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

 61 GCGTACGCTG AGGTGCAGCT GGTGGAGTCT GGGGGAGGCT TAGTGCCGCC TGGAGGGTCC
    CGCATGCGAC TCCACGTCGA CCACCTCAGA CCCCCTCCGA ATCACGGCGG ACCTCCCAGG
 -3 A  Y  A  E   V  Q  L   V  E  S   G  G  G  L   V  P  P   G  G  S

121 CTGAAACTCT CCTGTGCAGC CTCTGGATTC ATATTCAGTA GTTATGGCAT GTCTTGGGTT
    GACTTTGAGA GGACACGTCG GAGACCTAAG TATAAGTCAT CAATACCGTA CAGAACCCAA
 18 L  K  L  S   C  A  A   S  G  F   I  F  S  S   Y  G  M   S  W  V
                                               *   *  *  *   *
                                         CDR #1

181 CGCCAGACTC CAGGCAAGAG CCTGGAGTTG GTCGCAACCA TTAATAATAA TGGTGATAGC
    GCGGTCTGAG GTCCGTTCTC GGACCTCAAC CAGCGTTGGT AATTATTATT ACCACTATCG
 38 R  Q  T  P   G  K  S   L  E  L   V  A  T  I   N  N  N   G  D  S
                                          *  *   *  *  *   *  *  *

241 ACCTATTATC CAGACAGTGT GAAGGGCCGA TTCACCATCT CCCGAGACAA TGCCAAGAAC
    TGGATAATAG GTCTGTCACA CTTCCCGGCT AAGTGGTAGA GGGCTCTGTT ACGGTTCTTG
 58 T  Y  Y  P   D  S  V   K  G  R   F  T  I  S   R  D  N   A  K  N
    *  *  *  *   *  *  *   *
       CDR #2

301 ACCCTGTACC TGCAAATGAG CAGTCTGAAG TCTGAGGACA CAGCCATGTT TTACTGTGCA
    TGGGACATGG ACGTTTACTC GTCAGACTTC AGACTCCTGT GTCGGTACAA AATGACACGT
 78 T  L  Y  L   Q  M  S   S  L  K   S  E  D  T   A  M  F   Y  C  A

361 AGAGCCCTCA TTAGTTCGGC TACTTGGTTT GGTTACTGGG GCCAAGGGAC TCTGGTCACT
    TCTCGGGAGT AATCAAGCCG ATGAACCAAA CCAATGACCC CGGTTCCCTG AGACCAGTGA
 98 R  A  L  I   S  S  A   T  W  F   G  Y  W  G   Q  G  T   L  V  T
          *  *   *  *  *   *  *  *   *  *
                 CDR #3
                      ApaI
421 GTCTCTGCAG CCTCCACCAA GGGCCCATCG GTCTTCCCCC TGGCACCCTC CTCCAAGAGC
    CAGAGACGTC GGAGGTGGTT CCCGGGTAGC CAGAAGGGGG ACCGTGGGAG GAGGTTCTCG
118 V  S  A  A   S  T  K   G  P  S   V  F  P  L   A  P  S   S  K  S

481 ACCTCTGGGG GCACAGCGGC CCTGGGCTGC CTGGTCAAGG ACTACTTCCC CGAACCGGTG
    TGGAGACCCC CGTGTCGCCG GGACCCGACG GACCAGTTCC TGATGAAGGG GCTTGGCCAC
138 T  S  G  G   T  A  A   L  G  C   L  V  K  D   Y  F  P   E  P  V

541 ACGGTGTCGT GGAACTCAGG CGCCCTGACC AGCGGCGTGC ACACCTTCCC GGCTGTCCTA
    TGCCACAGCA CCTTGAGTCC GCGGGACTGG TCGCCGCACG TGTGGAAGGG CCGACAGGAT
158 T  V  S  W   N  S  G   A  L  T   S  G  V  H   T  F  P   A  V  L

601 CAGTCCTCAG GACTCTACTC CCTCAGCAGC GTGGTGACCG TGCCCTCCAG CAGCTTGGGC
    GTCAGGAGTC CTGAGATGAG GGAGTCGTCG CACCACTGGC ACGGGAGGTC GTCGAACCCG
178 Q  S  S  G   L  Y  S   L  S  S   V  V  T  V   P  S  S   S  L  G
```

FIG. 20A

```
661 ACCCAGACCT ACATCTGCAA CGTGAATCAC AAGCCCAGCA ACACCAAGGT GGACAAGAAA
    TGGGTCTGGA TGTAGACGTT GCACTTAGTG TTCGGGTCGT TGTGGTTCCA CCTGTTCTTT
198 T  Q  T  Y   I  C  N   V  N  H   K  P  S  N   T  K  V   D  K  K

721 GTTGAGCCCA AATCTTGTGA CAAAACTCAC ACATGA
    CAACTCGGGT TTAGAACACT GTTTTGAGTG TGTACT
218 V  E  P  K   S  C  D   K  T  H   T  O
```

FIG. 20B

Light Chain Primers:

MKLC-1, 22mer

5' CAGTCCAACTGTTCAGGACGCC 3'

MKLC-2, 22mer

5' GTGCTGCTCATGCTGTAGGTGC 3'

MKLC-3, 23mer

5' GAAGTTGATGTCTTGTGAGTGGC 3'

Heavy Chain Primers:

IGG2AC-1, 24mer

5' GCATCCTAGAGTCACCGAGGAGCC 3'

IGG2AC-2, 22mer

5' CACTGGCTCAGGGAAATAACCC 3'

IGG2AC-3, 22mer

5' GGAGAGCTGGGAAGGTGTGCAC 3'

FIG. 21

Light chain forward primer

6G4.light.Nsi 36-MER

```
5' CCAATGCATACGCT GAC ATC GTG ATG ACC CAG ACC CC 3'
                    T   T           T       T
                                    A       A
```

Light chain reverse primer

6G4.light.Mun 35-MER

5' AGA TGT CAA TTG CTC ACT GGA TGG TGG GAA GAT GG 3'

FIG. 22

Heavy chain forward primer

6G4.heavy.Mlu 32-MER

```
5' CAAACGCGTACGCT GAG ATC CAG CTG CAG CAG  3'
                        T       C
```

Heavy chain reverse primer

SL002B 39-MER

```
5' CGATGGCCCGG ATAGACCGATGGGGCTGTTGTTTTGGC  3'
                      T
                      A
                      G
```

FIG. 23

```
 70 G ATATCGTGAT GACACAGACA CCACTCTCCC TGCCTGTCAG TCTTGGAGAT
    C TATAGCACTA CTGTGTCTGT GGTGAGAGGG ACGGACAGTC AGAACCTCTA
  1 D   I  V  M   T  Q  T   P  L  S  L   P  V  S   L  G  D

121 CAGGCCTCCA TCTCTTGCAG ATCTAGTCAG AGCCTTGTAC ACGGTATTGG AAACACCTAT
    GTCCGGAGGT AGAGAACGTC TAGATCAGTC TCGGAACATG TGCCATAACC TTTGTGGATA
 18 Q  A  S  I   S  C  R   S  S  Q  S   L  V  H   G  I  G   N  T  Y
                       *   *  *  *  *   *  *  *   *  *  *   *  *  *
                                        CDR #1

181 TTACATTGGT ACCTGCAGAA GCCAGGCCAG TCTCCAAAGC TCCTGATCTA CAAAGTTTCC
    AATGTAACCA TGGACGTCTT CGGTCCGGTC AGAGGTTTCG AGGACTAGAT GTTTCAAAGG
 38 L  H  W  Y   L  Q  K   P  G  Q   S  P  K  L   L  I  Y   K  V  S
    *  *                                                    *  *  *
                                                            CDR #2

241 AACCGATTTT CTGGGGTCCC AGACAGGTTC AGTGGCAGTG GATCAGGGAC AGATTTCACA
    TTGGCTAAAA GACCCCAGGG TCTGTCCAAG TCACCGTCAC CTAGTCCCTG TCTAAAGTGT
 58 N  R  F  S   G  V  P   D  R  F   S  G  S  G   S  G  T   D  F  T
    *  *  *  *

301 CTCAGGATCA GCAGAGTGGA GGCTGAGGAT CTGGGACTTT ATTTCTGCTC TCAAAGTACA
    GAGTCCTAGT CGTCTCACCT CCGACTCCTA GACCCTGAAA TAAAGACGAG AGTTTCATGT
 78 L  R  I  S   R  V  E   A  E  D   L  G  L  Y   F  C  S   Q  S  T
                                                       *   *  *  *
                                                         CDR #3

361 CATGTTCCGC TCACGTTCGG TGCTGGGACC AAGCTGGAGC TGAAACGGGC TGATGCTGCA
    GTACAAGGCG AGTGCAAGCC ACGACCCTGG TTCGACCTCG ACTTTGCCCG ACTACGACGT
 98 H  V  P  L   T  F  G   A  G  T   K  L  E  L   K  R  A   D  A  A
    *  *  *  *   *

                                     MunI
421 CCAACTGTAT CCATCTTCCC ACCATCCAGT GAGCAATTGA
    GGTTGACATA GGTAGAAGGG TGGTAGGTCA CTCGTTAACT
118 P  T  V  S   I  F  P   P  S  S   E  Q  L  K
```

FIG. 24

```
 70 G AGATTCAGCT GCAGCAGTCT GGACCTGAGC TGATGAAGCC TGGGGCTTCA
    C TCTAAGTCGA CGTCGTCAGA CCTGGACTCG ACTACTTCGG ACCCCGAAGT
  1 E  I  Q  L   Q  Q  S   G  P  E  L   M  K  P   G  A  S

121 GTGAAGATAT CCTGCAAGGC TTCTGGTTAT TCATTCAGTA GCCACTACAT GCACTGGGTG
    CACTTCTATA GGACGTTCCG AAGACCAATA AGTAAGTCAT CGGTGATGTA CGTGACCCAC
 18 V  K  I  S   C  K  A   S  G  Y   S  F  S  S  H   Y  M   H  W  V
                                                *  *  *  *   *
                                          CDR #1

181 AAGCAGAGCC ATGGAAAGAG CCTTGAGTGG ATTGGCTACA TTGATCCTTC CAATGGTGAA
    TTCGTCTCGG TACCTTTCTC GGAACTCACC TAACCGATGT AACTAGGAAG GTTACCACTT
 38 K  Q  S  H   G  K  S   L  E  W   I  G  Y  I   D  P  S   N  G  E
                                          *  *   *  *  *   *  *  *
                                                 CDR #2

241 ACTACTTACA ACCAGAAATT CAAGGGCAAG GCCACATTGA CTGTAGACAC ATCTTCCAGC
    TGATGAATGT TGGTCTTTAA GTTCCCGTTC CGGTGTAACT GACATCTGTG TAGAAGGTCG
 58 T  T  Y  N   Q  K  F   K  G  K   A  T  L  T   V  D  T   S  S  S
    *  *  *  *   *  *  *   *  *

301 ACAGCCAACG TGCATCTCAG CAGCCTGACA TCTGATGACT CTGCAGTCTA TTTCTGTGCA
    TGTCGGTTGC ACGTAGAGTC GTCGGACTGT AGACTACTGA GACGTCAGAT AAAGACACGT
 78 T  A  N  V   H  L  S   S  L  T   S  D  D  S   A  V  Y   F  C  A

361 AGAGGGGACT ATAGATACAA CGGCGACTGG TTTTTCGATG TCTGGGGCGC AGGGACCACG
    TCTCCCCTGA TATCTATGTT GCCGCTGACC AAAAAGCTAC AGACCCCGCG TCCCTGGTGC
 98 R  G  D  Y   R  Y  N   G  D  W   F  F  D   V   W  G  A   G  T  T
       *  *  *   *  *  *   *  *  *   *  *  *   *
                 CDR #3
  BstEII                                         ApaI
421 GTCACCGTCT CCTCCGCCAA AACCGACAGC CCCATCGGTC TATCCGGGCC
    CAGTGGCAGA GGAGGCGGAT TTGGCTGTCG GGGTAGCCAG ATAGGCCCGG
118 V  T  V  S   S  A  K   T  D  S   P  I  G  L   S  G  P

471 CATC
    GTAG
135  I
```

FIG. 25

5' CTTGGTGGAGGCGGAGGAGACG 3'

Mutagenesis Primer for 6G425VL

DS/VF 38MER

5' GAAACGGGCTGTTGCTGCACCAACTGTATTCATCTTCC 3'

SYN.BstEII 31 MER

5' GTCACCGTCT CCTCCGCCTC CACCAAGGGC C 3'

SYN.Apa 22 MER

5' CTTGGTGGAGGCGGAGGAGACG 3'

FIG. 26

```
  1 ATGAAGAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAT
    TACTTCTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTA
-23 M  K  K  N    I  A  F    L  L  A    S  M  F  V    F  S  I    A  T  N

 61 GCATACGCTG ATATCGTGAT GACACAGACA CCACTCTCCC TGCCTGTCAG TCTTGGAGAT
    CGTATGCGAC TATAGCACTA CTGTGTCTGT GGTGAGAGGG ACGGACAGTC AGAACCTCTA
 -3 A  Y  A  D    I  V  M    T  Q  T    P  L  S  L    P  V  S    L  G  D

121 CAGGCCTCCA TCTCTTGCAG ATCTAGTCAG AGCCTTGTAC ACGGTATTGG AAACACCTAT
    GTCCGGAGGT AGAGAACGTC TAGATCAGTC TCGGAACATG TGCCATAACC TTTGTGGATA
 18 Q  A  S  I    S  C  R    S  S  Q    S  L  V  H    G  I  G    N  T  Y
                        *    *  *  *    *  *  *  *    *  *  *    *  *  *
                                       CDR #1

181 TTACATTGGT ACCTGCAGAA GCCAGGCCAG TCTCCAAAGC TCCTGATCTA CAAAGTTTCC
    AATGTAACCA TGGACGTCTT CGGTCCGGTC AGAGGTTTCG AGGACTAGAT GTTTCAAAGG
 38 L  H  W  Y    L  Q  K    P  G  Q    S  P  K  L    L  I  Y    K  V  S
    *  *                                                         *  *  *
                                                                CDR #2

241 AACCGATTTT CTGGGGTCCC AGACAGGTTC AGTGGCAGTG GATCAGGGAC AGATTTCACA
    TTGGCTAAAA GACCCCAGGG TCTGTCCAAG TCACCGTCAC CTAGTCCCTG TCTAAAGTGT
 58 N  R  F  S    G  V  P    D  R  F    S  G  S  G    S  G  T    D  F  T
    *  *  *  *

301 CTCAGGATCA GCAGAGTGGA GGCTGAGGAT CTGGGACTTT ATTTCTGCTC TCAAAGTACA
    GAGTCCTAGT CGTCTCACCT CCGACTCCTA GACCCTGAAA TAAAGACGAG AGTTTCATGT
 78 L  R  I  S    R  V  E    A  E  D    L  G  L  Y    F  C  S    Q  S  T
                                                            *    *  *  *
                                                              CDR #3

361 CATGTTCCGC TCACGTTCGG TGCTGGGACC AAGCTGGAGC TGAAACGGGC TGTTGCTGCA
    GTACAAGGCG AGTGCAAGCC ACGACCCTGG TTCGACCTCG ACTTTGCCCG ACAACGACGT
 98 H  V  P  L    T  F  G    A  G  T    K  L  E  L    K  R  A    V  A  A
    *  *  *  *    *

421 CCAACTGTAT TCATCTTCCC ACCATCCAGT GAGCAATTGA AATCTGGAAC TGCCTCTGTT
    GGTTGACATA AGTAGAAGGG TGGTAGGTCA CTCGTTAACT TTAGACCTTG ACGGAGACAA
118 P  T  V  F    I  F  P    P  S  S    E  Q  L  K    S  G  T    A  S  V

481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC
    CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG
138 V  C  L  L    N  N  F    Y  P  R    E  A  K  V    Q  W  K    V  D  N

541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC
    CGGGAGGTTA GCCCATTGAG GGTCCTCTCA CAGTGTCTCG TCCTGTCGTT CCTGTCGTGG
158 A  L  Q  S    G  N  S    Q  E  S    V  T  E  Q    D  S  K    D  S  T

601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC
    ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG
178 Y  S  L  S    S  T  L    T  L  S    K  A  D  Y    E  K  H    K  V  Y
```

FIG. 27A

```
661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA
    CGGACGCTTC AGTGGGTAGT CCCGGACTCG AGCGGGCAGT GTTTCTCGAA GTTGTCCCCT
198 A  C  E  V   T  H  Q   G  L  S   S  P  V  T   K  S  F   N  R  G

721 GAGTGTTAA
    CTCACAATT
218 E  C  O
```

FIG. 27B

```
  1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

 61 GCGTACGCTG AGATTCAGCT GCAGCAGTCT GGACCTGAGC TGATGAAGCC TGGGGCTTCA
    CGCATGCGAC TCTAAGTCGA CGTCGTCAGA CCTGGACTCG ACTACTTCGG ACCCCGAAGT
 -3 A  Y  A  E   I  Q  L   Q  Q  S   G  P  E  L   M  K  P   G  A  S

121 GTGAAGATAT CCTGCAAGGC TTCTGGTTAT TCATTCAGTA GCCACTACAT GCACTGGGTG
    CACTTCTATA GGACGTTCCG AAGACCAATA AGTAAGTCAT CGGTGATGTA CGTGACCCAC
 18 V  K  I  S   C  K  A   S  G  Y   S  F  S  S   H  Y  M   H  W  V
                                           *   *  *  *   *
                                      CDR #1

181 AAGCAGAGCC ATGGAAAGAG CCTTGAGTGG ATTGGCTACA TTGATCCTTC CAATGGTGAA
    TTCGTCTCGG TACCTTTCTC GGAACTCACC TAACCGATGT AACTAGGAAG GTTACCACTT
 38 K  Q  S  H   G  K  S   L  E  W   I  G  Y  I   D  P  S   N  G  E
                                       *  *   *  *  *   *  *  *
                                            CDR #2

241 ACTACTTACA ACCAGAAATT CAAGGGCAAG GCCACATTGA CTGTAGACAC ATCTTCCAGC
    TGATGAATGT TGGTCTTTAA GTTCCCGTTC CGGTGTAACT GACATCTGTG TAGAAGGTCG
 58 T  T  Y  N   Q  K  F   K  G  K   A  T  L  T   V  D  T   S  S  S
    *  *  *  *   *  *  *   *  *

301 ACAGCCAACG TGCATCTCAG CAGCCTGACA TCTGATGACT CTGCAGTCTA TTTCTGTGCA
    TGTCGGTTGC ACGTAGAGTC GTCGGACTGT AGACTACTGA GACGTCAGAT AAAGACACGT
 78 T  A  N  V   H  L  S   S  L  T   S  D  D  S   A  V  Y   F  C  A

361 AGAGGGGACT ATAGATACAA CGGCGACTGG TTTTTCGATG TCTGGGGCGC AGGGACCACG
    TCTCCCCTGA TATCTATGTT GCCGCTGACC AAAAAGCTAC AGACCCCGCG TCCCTGGTGC
 98 R  G  D  Y   R  Y  N   G  D  W   F  F  D  V   W  G  A   G  T  T
       *  *  *   *  *  *   *  *  *   *  *  *  *
            CDR #3

421 GTCACCGTCT CCTCCGCCTC CACCAAGGGC CCATCGGTCT TCCCCCTGGC ACCCTCCTCC
    CAGTGGCAGA GGAGGCGGAG GTGGTTCCCG GGTAGCCAGA AGGGGGACCG TGGGAGGAGG
118 V  T  V  S   S  A  S   T  K  G   P  S  V  F   P  L  A   P  S  S

481 AAGAGCACCT CTGGGGGCAC AGCGGCCCTG GGCTGCCTGG TCAAGGACTA CTTCCCCGAA
    TTCTCGTGGA GACCCCCGTG TCGCCGGGAC CCGACGGACC AGTTCCTGAT GAAGGGGCTT
138 K  S  T  S   G  G  T   A  A  L   G  C  L  V   K  D  Y   F  P  E

541 CCGGTGACGG TGTCGTGGAA CTCAGGCGCC CTGACCAGCG GCGTGCACAC CTTCCCGGCT
    GGCCACTGCC ACAGCACCTT GAGTCCGCGG GACTGGTCGC CGCACGTGTG GAAGGGCCGA
158 P  V  T  V   S  W  N   S  G  A   L  T  S  G   V  H  T   F  P  A

601 GTCCTACAGT CCTCAGGACT CTACTCCCTC AGCAGCGTGG TGACCGTGCC CTCCAGCAGC
    CAGGATGTCA GGAGTCCTGA GATGAGGGAG TCGTCGCACC ACTGGCACGG GAGGTCGTCG
178 V  L  Q  S   S  G  L   Y  S  L   S  S  V  V   T  V  P   S  S  S
```

FIG. 28A

```
661 TTGGGCACCC AGACCTACAT CTGCAACGTG AATCACAAGC CCAGCAACAC CAAGGTGGAC
    AACCCGTGGG TCTGGATGTA GACGTTGCAC TTAGTGTTCG GGTCGTTGTG GTTCCACCTG
198 L  G  T  Q   T  Y  I   C  N  V   N  H  K  P   S  N  T   K  V  D

721 AAGAAAGTTG AGCCCAAATC TTGTGACAAA ACTCACACAT GA
    TTCTTTCAAC TCGGGTTTAG AACACTGTTT TGAGTGTGTA CT
218 K  K  V  E   P  K  S   C  D  K   T  H  T  O
```

FIG. 28B

Variable Light Chain Domain

```
                  10        20       abcde 30        40
6G425     DIVMTQTPLSLPVSLGDQASISCRSSQSLVHGIGNTYLHWYLQKPGQSPKLLIY
            #   # #  ## #  ### #                   #    ##
F(ab)-1   DIQMTQSPSSLSASVGDRVTITCRSSQSLVHGIGNTYLHWYQQKPGKAPKLLIY
                                  # ##########
humκI     DIQMTQSPSSLSASVGDRVTITCRASKTI-----SKYLAWYQQKPGKAPKLLIY
                                   ============
                                +++++++++++++++++
                                         L1

          50        60        70        80        90        100
6G425     YKVSNRFSGVPDRFSDSGSGTDFTLRISRVEAEDLGLYFCSQSTHVPLTFGAGTKLELKR
                     #   #         #  ##### ### #            #   # #
F(ab)-1   YKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCSQSTHVPLTFGQGTKVEIKR
           ## ###                                 # ####
humκI     YSGSTLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQGTKVEIKR
           ===                                      ======
           +++++++                                +++++++++
               L2                                      L3
```

Variable Heavy Chain Domain

```
                  10        20        30        40
6G425     EIQLQQSGPELMKPGASVKISCKASGYSFSSHYMHWVKQSHGKSLEWI
           #  ##  ## ##  # ###  #              # ##  #   #
F(ab)-1   EVQLVESGGGLVQPGGSLRLSCAASGYSFSSHYMHWVRQAPGKGLEWV
                                    #  ## # #
humIII    EVQLVESGGGLVQPGGSLRLSCAASGFSFTGHWMNWVRQAPGKGLEWV
                                    =======
                                         +++++
                                        H1

          50  a                70        80  abc      90        100      110
6G425     GYIDPSNGETTYNQKFKGKATLTVDTSSSTANVHLSSLTSDDSAVYFCAARGDYRYNGDWFFDVWGAGT
                            ## ### # ## ######  ### #   #                   #
F(ab)-1   GYIDPSNGETTYNQKFKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAARGDYRYNGDWFFDVWGQGT
           # #  ##  # ####                                    # # # ###  #
humIII    GMIHPSDSETRYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAARGIYFY-GTTYFDYWGQGT
              ====                                            ===========
           ++++++++++++++++++                                +++++++++++++
                 H2                                                H3
```

FIG. 29

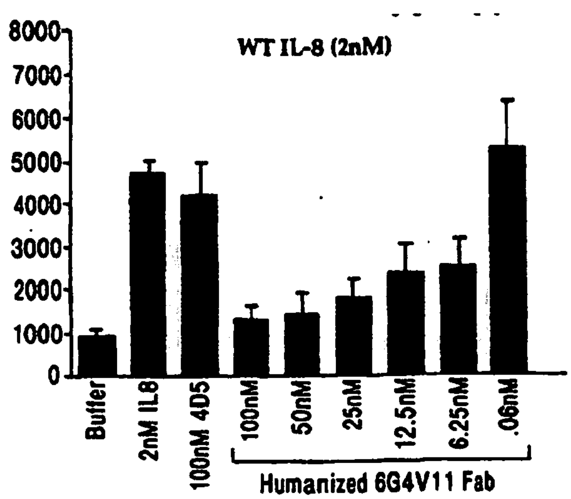


FIG. 30A

IC50~12nM

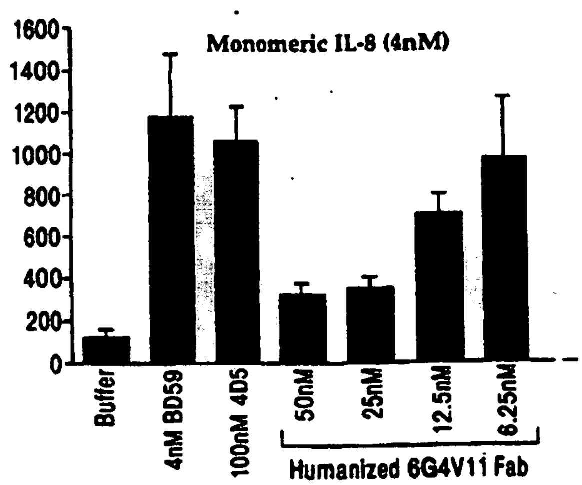


FIG. 30B

IC50~15nM

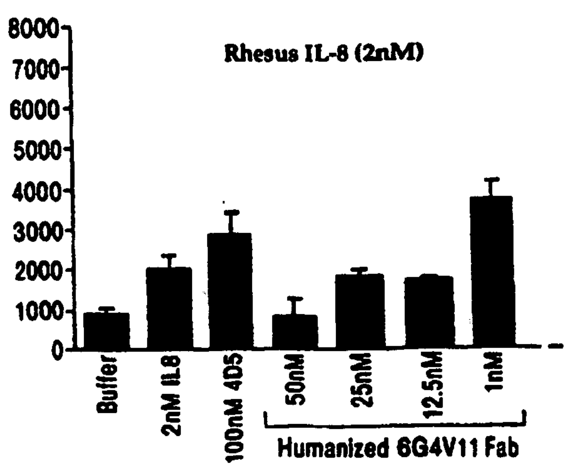


FIG. 30C

IC50~22nM

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11 Light Chain

MKKNIAFLLASMFVFSIATNAYADIQMTQSPSSLSASVGDRVTITCRSSQSLVHGIGNTY
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCSQST
HVPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG
EC

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11 Heavy Chain

MKKNIAFLLASMFVFSIATNAYAEVQLVQSGGGLVQPGGSLRLSCAASGYSFSSHYMH
WVRQAPGKGLEWVGYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY
CARGDYRYNGDWFFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
VDKKVEPKSCDKTHT

Amino Acid Sequence of the peptide linker and M13 Phage Coat (gene-III)

SGGGSGSGDFDYEKMANANKGAMTENADENALQSDAKGKLDSVATDYGAAIDGFIGDVS
GLANGNGATGDFAGSSNSQMAQVGDGDNSPLMNNFRQYLPSLPQSVECRPFVFSAGKPY
EFSIDCDKINLFRGVFAFLLYVATFMYVFSTFANILRNKES

FIG. 31A

```
  1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

 61 GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCCGCCTC TGTGGGCGAT
    CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA
 -3 A  Y  A  D   I  Q  M   T  Q  S   P  S  S  L   S  A  S   V  G  D

121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TAACACGTAT
    TCCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACGATGCATA
 18 R  V  T  I   T  C  R   S  S  Q   S  L  V  H   G  I  G   N  T  Y

181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC
    AATGTGACCA TAGTTGTCTT TGGTCCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG
 38 L  H  W  Y   Q  Q  K   P  G  K   A  P  K  L   L  I  Y   K  V  S

241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT
    TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCTG CCTAAAGTGA
 58 N  R  F  S   G  V  P   S  R  F   S  G  S  G   S  G  T   D  F  T

301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT
    GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA
 78 L  T  I  S   S  L  Q   P  E  D   F  A  T  Y   Y  C  S   Q  S  T

361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA
    GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT
 98 H  V  P  L   T  F  G   Q  G  T   K  V  E  I   K  R  T   V  A  A

421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT
    GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA
118 P  S  V  F   I  F  P   P  S  D   E  Q  L  K   S  G  T   A  S  V

481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC
    CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG
138 V  C  L  L   N  N  F   Y  P  R   E  A  K  V   Q  W  K   V  D  N

541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC
    CGGGAGGTTA GCCCATTGAG GGTCCTCTCA CAGTGTCTCG TCCTGTCGTT CCTGTCGTGG
158 A  L  Q  S   G  N  S   Q  E  S   V  T  E  Q   D  S  K   D  S  T

601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC
    ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG
178 Y  S  L  S   S  T  L   T  L  S   K  A  D  Y   E  K  H   K  V  Y

661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA
    CGGACGCTTC AGTGGGTAGT CCCGGACTCG AGCGGGCAGT GTTTCTCGAA GTTGTCCCCT
198 A  C  E  V   T  H  Q   G  L  S   S  P  V  T   K  S  F   N  R  G

721 GAGTGTTAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA
    CTCACAATTC GACTAGGAGA TGCGGCCTGC GTAGCACCGG GATCATGCGT TGATCAGCAT
218 E  C  O
```

FIG. 31B

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V19 Light Chain

MKKNIAFLLASMFVFSIATNAYADIQMTQSPSSLSASVGDRVTITCRSSQSLVHGIGNTY
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCSQST
HVPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG
EC

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V19 Heavy Chain

MKKNIAFLLASMFVFSIATNAYAEVQLVESGGGLVQPGGSLRLSCAASGYSFSSHYMH
WVKQAPGKGLEWVGYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY
CARGDYRYNGDWFFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
VDKKVEPKSCDKTHT

FIG. 31C

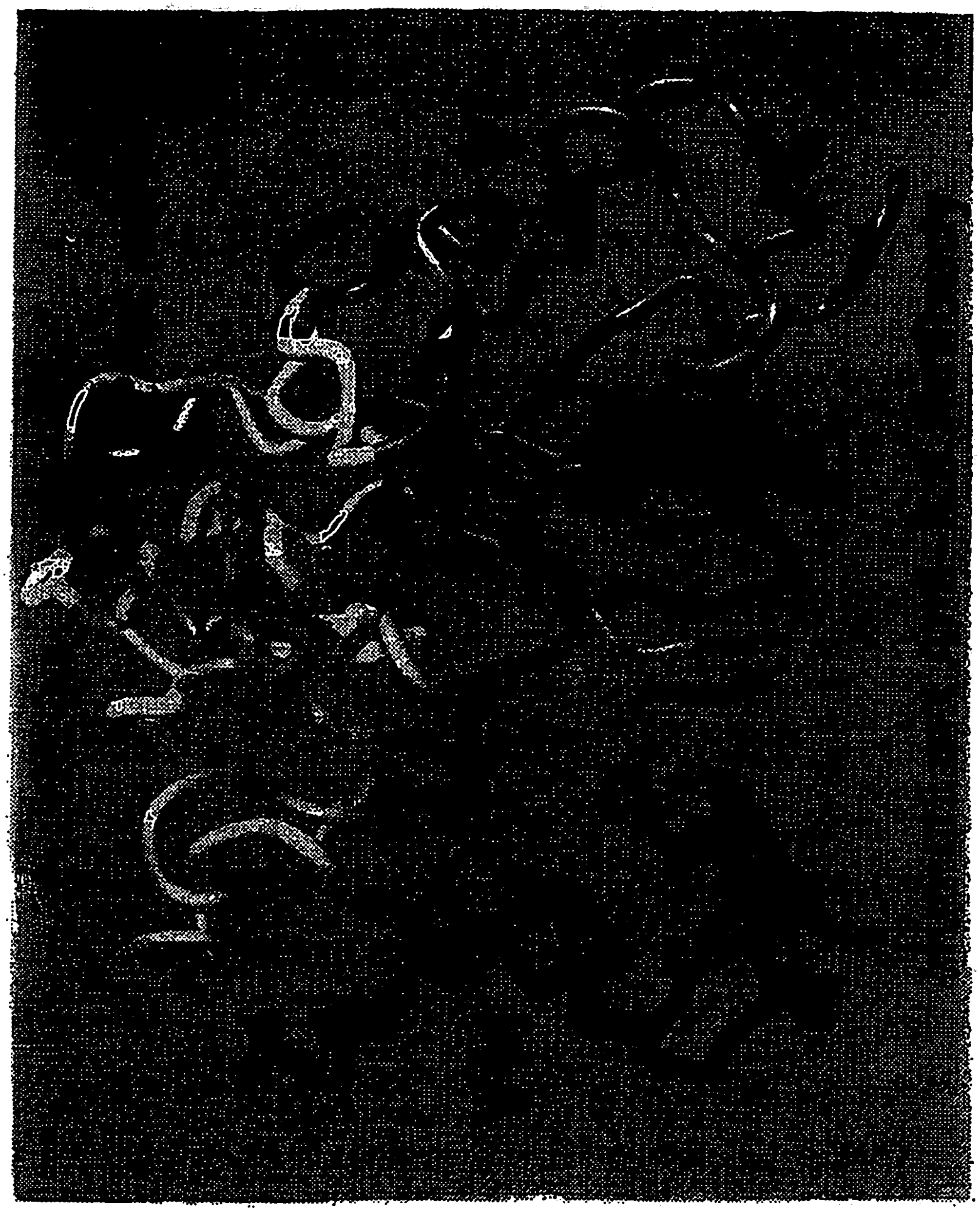


FIG.32

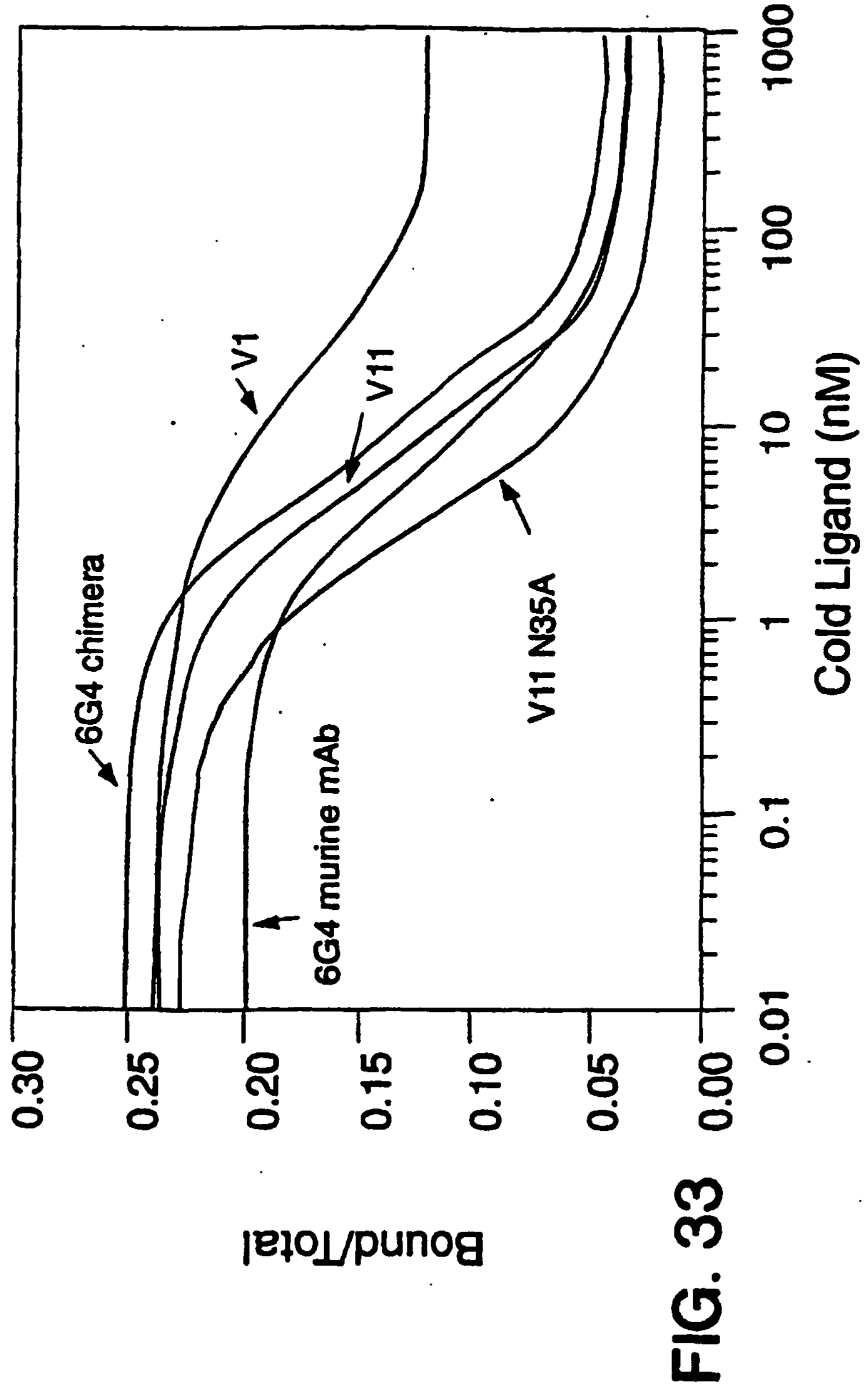

Bound/Total
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.01
0.1
1
10
100
1000
Cold Ligand (nM)
6G4 chimera
6G4 murine mAb
V1
V11
V11 N35A

FIG. 33

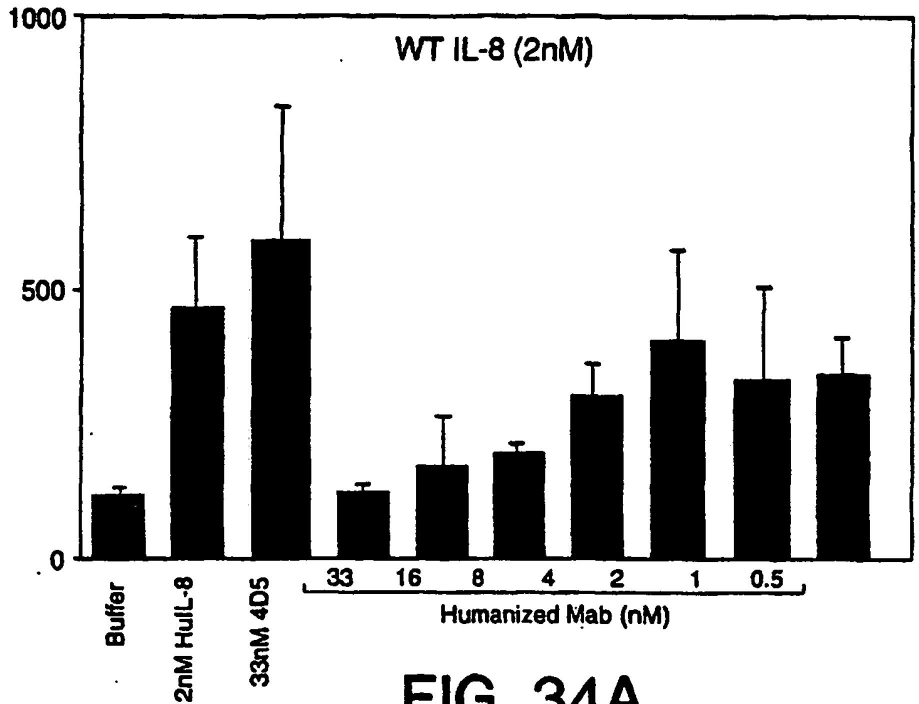


FIG. 34A

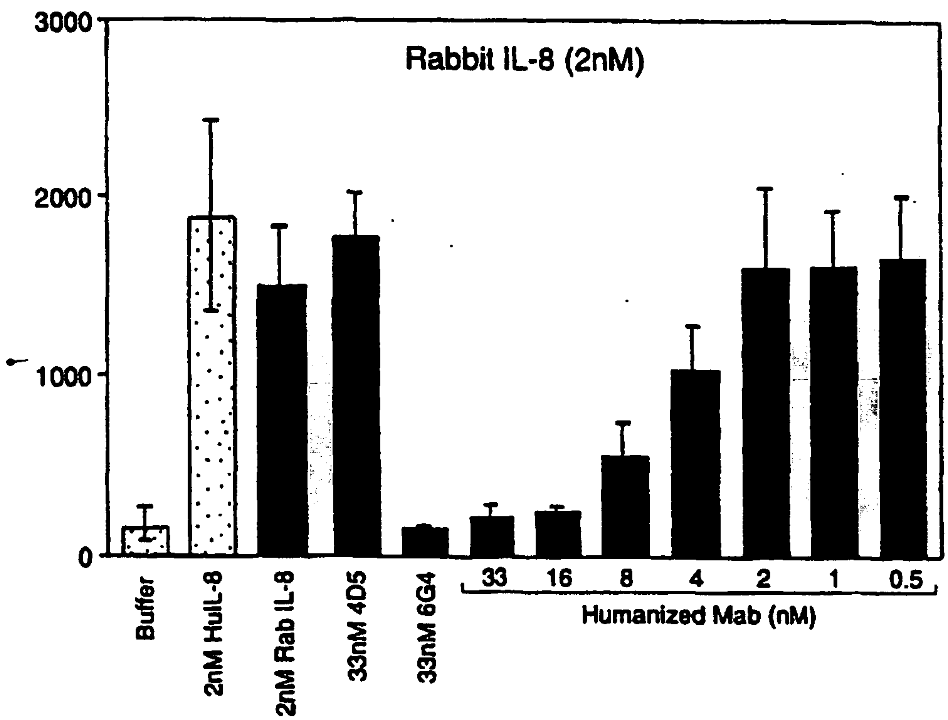


FIG. 34B

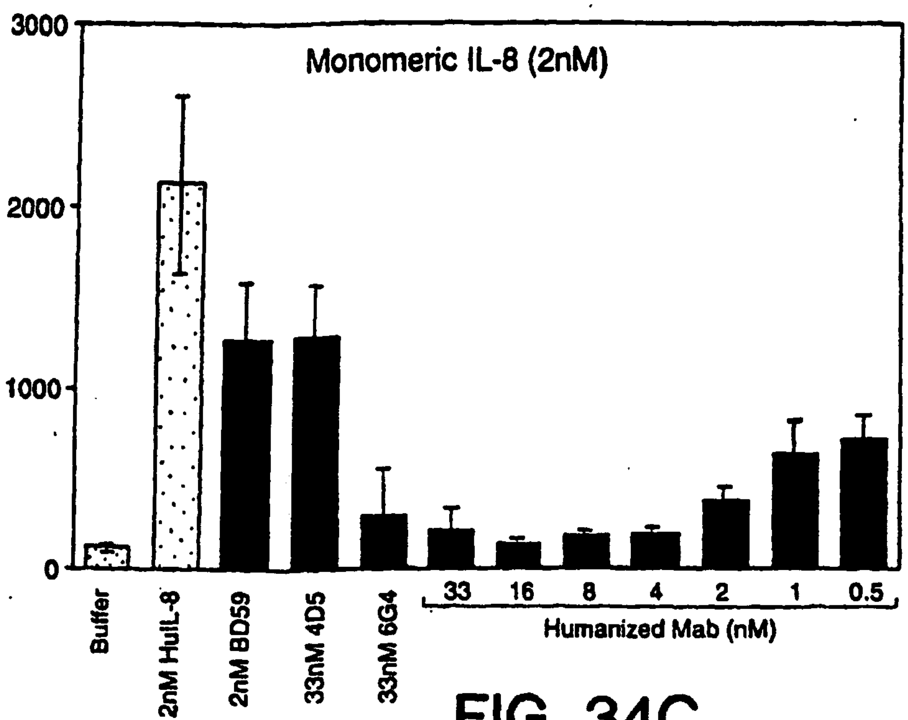


FIG. 34C

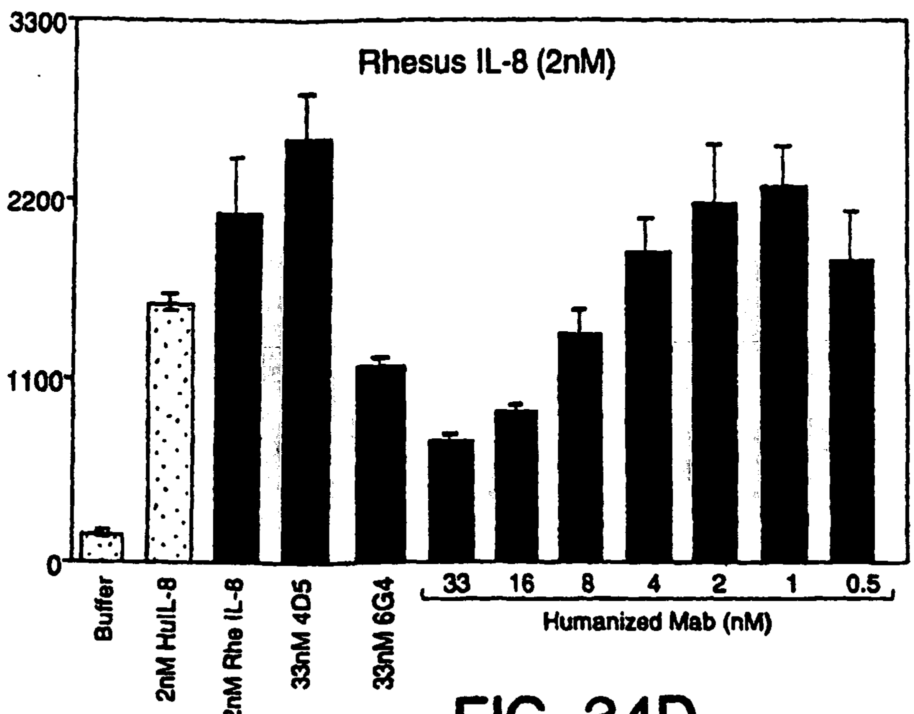


FIG. 34D

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11N35A Light Chain

MKKNIAFLLASMFVFSIATNAYADIQMTQSPSSLSASVGDRVTITCRSSQSLVHGIGATY
LHWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCSQST
HVPLTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN
ALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRG
EC

Amino Acid Sequence of the humanized anti-IL-8 6G4.2.5V11N35A Heavy Chain

MKKNIAFLLASMFVFSIATNAYAEVQLVQSGGGLVQPGGSLRLSCAASGYSFSSHYMH
WVRQAPGKGLEWVGYIDPSNGETTYNQKFKGRFTLSRDNSKNTAYLQMNSLRAEDTAVYY
CARGDYRYNGDWFFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK
VDKKVEPKSCDKTHT

Amino Acid Sequence of the putative Pepsin Cleavage Site and GCN4 Leucine Zipper

CPPCPAPELLGGRMKQLEDKVEELLSKNYHLENEVARLKKLVGER

FIG. 35

```
1   ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

61  GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCCGCCTC TGTGGGCGAT
    CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA
-3  A  Y  A  D   I  Q  M   T  Q  S   P  S  S  L   S  A  S   V  G  D

121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TGCTACGTAT
    TCCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACGATGCATA
18  R  V  T  I   T  C  R   S  S  Q   S  L  V  H   G  I  G   A  T  Y

181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC
    AATGTGACCA TAGTTGTCTT TGGTCCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG
38  L  H  W  Y   Q  Q  K   P  G  K   A  P  K  L   L  I  Y   K  V  S

241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT
    TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCTG CCTAAAGTGA
58  N  R  F  S   G  V  P   S  R  F   S  G  S  G   S  G  T   D  F  T

301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT
    GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA
78  L  T  I  S   S  L  Q   P  E  D   F  A  T  Y   Y  C  S   Q  S  T

361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA
    GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT
98  H  V  P  L   T  F  G   Q  G  T   K  V  E  I   K  R  T   V  A  A

421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT
    GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA
118 P  S  V  F   I  F  P   P  S  D   E  Q  L  K   S  G  T   A  S  V

481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC
    CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG
138 V  C  L  L   N  N  F   Y  P  R   E  A  K  V   Q  W  K   V  D  N

541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC
    CGGGAGGTTA GCCCATTGAG GGTCCTCTCA CAGTGTCTCG TCCTGTCGTT CCTGTCGTGG
158 A  L  Q  S   G  N  S   Q  E  S   V  T  E  Q   D  S  K   D  S  T

601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC
    ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG
178 Y  S  L  S   S  T  L   T  L  S   K  A  D  Y   E  K  H   K  V  Y

661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA
    CGGACGCTTC AGTGGGTAGT CCCGGACTCG AGCGGGCAGT GTTTCTCGAA GTTGTCCCCT
198 A  C  E  V   T  H  Q   G  L  S   S  P  V  T   K  S  F   N  R  G

721 GAGTGTTAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA
    CTCACAATTC GACTAGGAGA TGCGGCCTGC GTAGCACCGG GATCATGCGT TGATCAGCAT
218 E  C  O
```

FIG. 36

```
781  AAAAGGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT TCTTCTTGCA
     TTTTCCCATA GATCTCCAAC TCCACTAAAA TACTTTTTCT TATAGCGTAA AGAAGAACGT
-1                                    M  K  K  N   I  A  F   L  L  A

841  TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG AGGTTCAGCT AGTGCAGTCT
     AGATACAAGC AAAAAAGATA ACGATGTTTG CGCATGCGAC TCCAAGTCGA TCACGTCAGA
-11  S  M  F  V   F  S  I   A  T  N   A  Y  A  E   V  Q  L   V  Q  S

901  GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA CTCCGTTTGT CCTGTGCAGC TTCTGGCTAC
     CCGCCACCGG ACCACGTCGG TCCCCCGAGT GAGGCAAACA GGACACGTCG AAGACCGATG
8    G  G  G  L   V  Q  P   G  G  S   L  R  L  S   C  A  A   S  G  Y

961  TCCTTCTCGA GTCACTATAT GCACTGGGTC CGTCAGGCCC CGGGTAAGGG CCTGGAATGG
     AGGAAGAGCT CAGTGATATA CGTGACCCAG GCAGTCCGGG GCCCATTCCC GGACCTTACC
28   S  F  S  S   H  Y  M   H  W  V   R  Q  A  P   G  K  G   L  E  W

1021 GTTGGATATA TTGATCCTTC CAATGGTGAA ACTACGTATA ATCAAAAGTT CAAGGGCCGT
     CAACCTATAT AACTAGGAAG GTTACCACTT TGATGCATAT TAGTTTTCAA GTTCCCGGCA
48   V  G  Y  I   D  P  S   N  G  E   T  T  Y  N   Q  K  F   K  G  R

1081 TTCACTTTAT CTCGCGACAA CTCCAAAAAC ACAGCATACC TGCAGATGAA CAGCCTGCGT
     AAGTGAAATA GAGCGCTGTT GAGGTTTTTG TGTCGTATGG ACGTCTACTT GTCGGACGCA
68   F  T  L  S   R  D  N   S  K  N   T  A  Y  L   Q  M  N   S  L  R

1141 GCTGAGGACA CTGCCGTCTA TTACTGTGCA AGAGGGGATT ATCGCTACAA TGGTGACTGG
     CGACTCCTGT GACGGCAGAT AATGACACGT TCTCCCCTAA TAGCGATGTT ACCACTGACC
88   A  E  D  T   A  V  Y   Y  C  A   R  G  D  Y   R  Y  N   G  D  W

1201 TTCTTCGACG TCTGGGGTCA AGGAACCCTG GTCACCGTCT CCTCGGCCTC CACCAAGGGC
     AAGAAGCTGC AGACCCCAGT TCCTTGGGAC CAGTGGCAGA GGAGCCGGAG GTGGTTCCCG
108  F  F  D  V   W  G  Q   G  T  L   V  T  V  S   S  A  S   T  K  G

1261 CCATCGGTCT TCCCCCTGGC ACCCTCCTCC AAGAGCACCT CTGGGGGCAC AGCGGCCCTG
     GGTAGCCAGA AGGGGGACCG TGGGAGGAGG TTCTCGTGGA GACCCCCGTG TCGCCGGGAC
128  P  S  V  F   P  L  A   P  S  S   K  S  T  S   G  G  T   A  A  L

1321 GGCTGCCTGG TCAAGGACTA CTTCCCCGAA CCGGTGACGG TGTCGTGGAA CTCAGGCGCC
     CCGACGGACC AGTTCCTGAT GAAGGGGCTT GGCCACTGCC ACAGCACCTT GAGTCCGCGG
148  G  C  L  V   K  D  Y   F  P  E   P  V  T  V   S  W  N   S  G  A

1381 CTGACCAGCG GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC
     GACTGGTCGC CGCACGTGTG GAAGGGCCGA CAGGATGTCA GGAGTCCTGA GATGAGGGAG
168  L  T  S  G   V  H  T   F  P  A   V  L  Q  S   S  G  L   Y  S  L

1441 AGCAGCGTGG TGACCGTGCC CTCCAGCAGC TTGGGCACCC AGACCTACAT CTGCAACGTG
     TCGTCGCACC ACTGGCACGG GAGGTCGTCG AACCCGTGGG TCTGGATGTA GACGTTGCAC
188  S  S  V  V   T  V  P   S  S  S   L  G  T  Q   T  Y  I   C  N  V

1501 AATCACAAGC CCAGCAACAC CAAGGTCGAC AAGAAAGTTG AGCCCAAATC TTGTGACAAA
     TTAGTGTTCG GGTCGTTGTG GTTCCAGCTG TTCTTTCAAC TCGGGTTTAG AACACTGTTT
208  N  H  K  P   S  N  T   K  V  D   K  K  V  E   P  K  S   C  D  K

1561 ACTCACACAT GCCCGCCGTG CCCAGCACCA GAACTGCTGG GCGGCCGCAT GAAACAGCTA
     TGAGTGTGTA CGGGCGGCAC GGGTCGTGGT CTTGACGACC CGCCGGCGTA CTTTGTCGAT
228  T  H  T  C   P  P  C   P  A  P   E  L  L  G   G  R  M   K  Q  L
```

FIG. 37A

```
1621 GAGGACAAGG TCGAAGAGCT ACTCTCCAAG AACTACCACC TAGAGAATGA AGTGGCAAGA
     CTCCTGTTCC AGCTTCTCGA TGAGAGGTTC TTGATGGTGG ATCTCTTACT TCACCGTTCT
 248 E  D  K  V   E  E  L   L  S  K   N  Y  H  L   E  N  E   V  A  R

1681 CTCAAAAAGC TTGTCGGGGA GCGCTAA
     GAGTTTTTCG AACAGCCCCT CGCGATT
 268 L  K  K  L   V  G  E   R  O
```

FIG. 37B

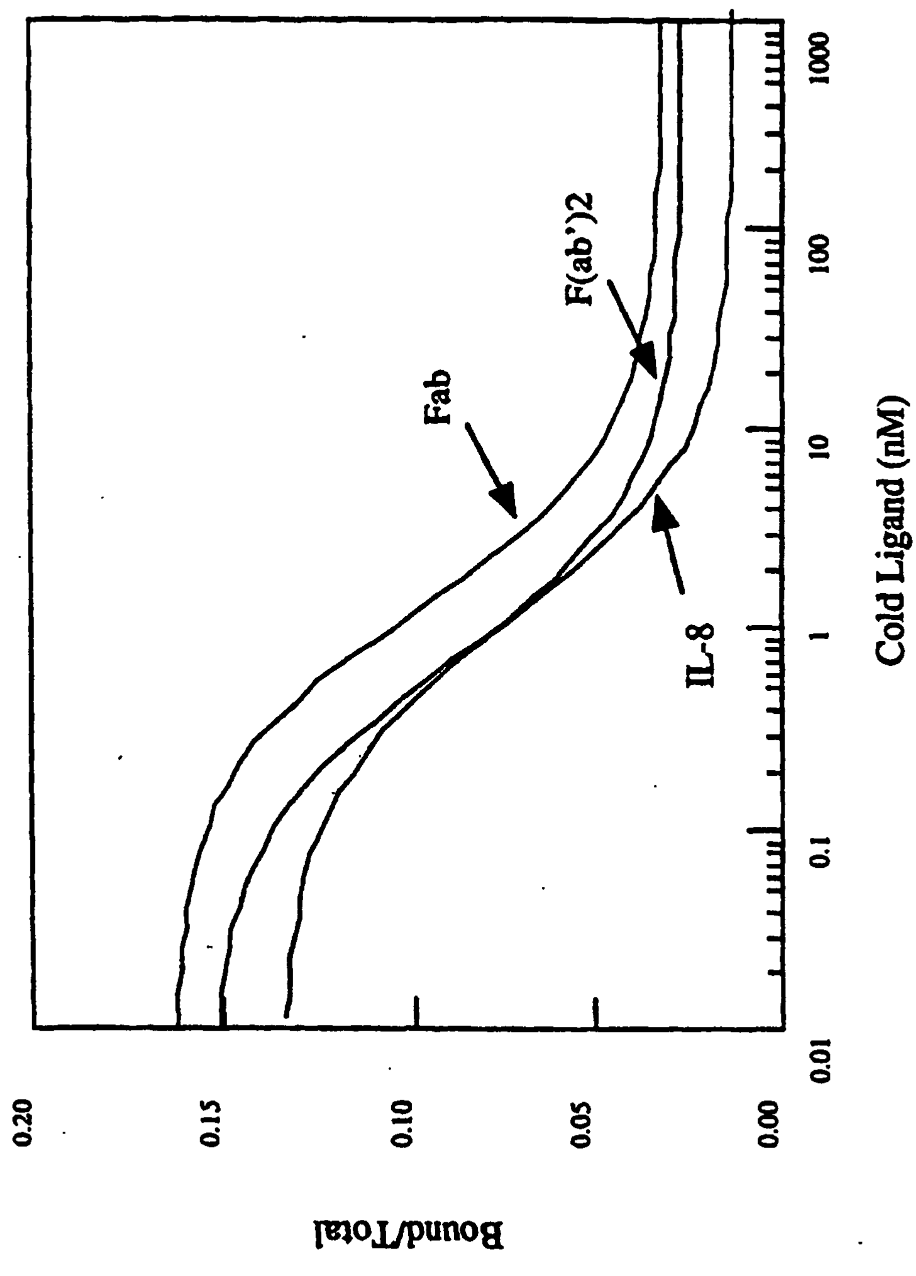

Bound/Total
0.20
0.15
0.10
0.05
0.00
0.01
0.1
1
10
100
1000
Cold Ligand (nM)
Fab
F(ab')2
IL-8

FIG. 38

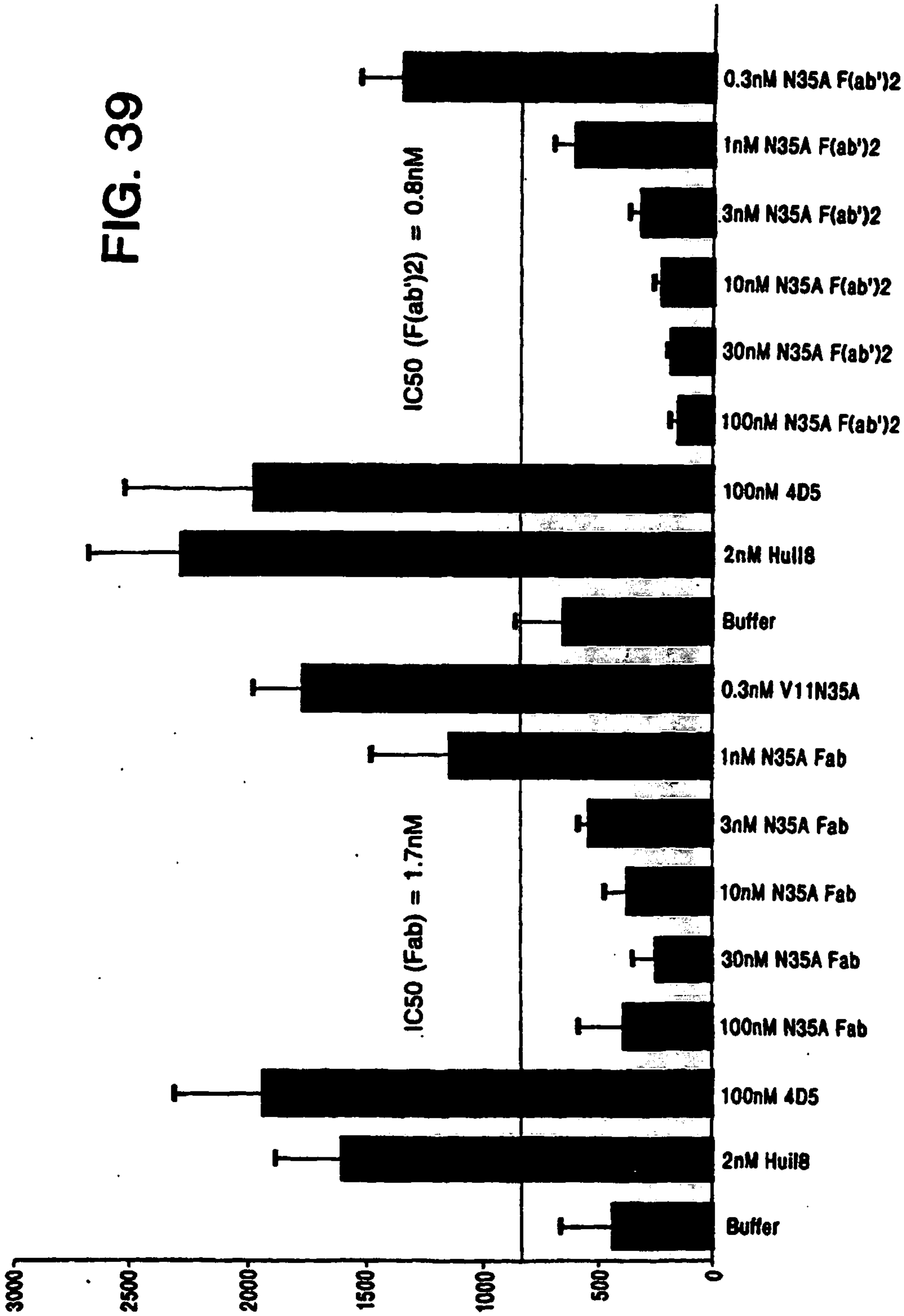
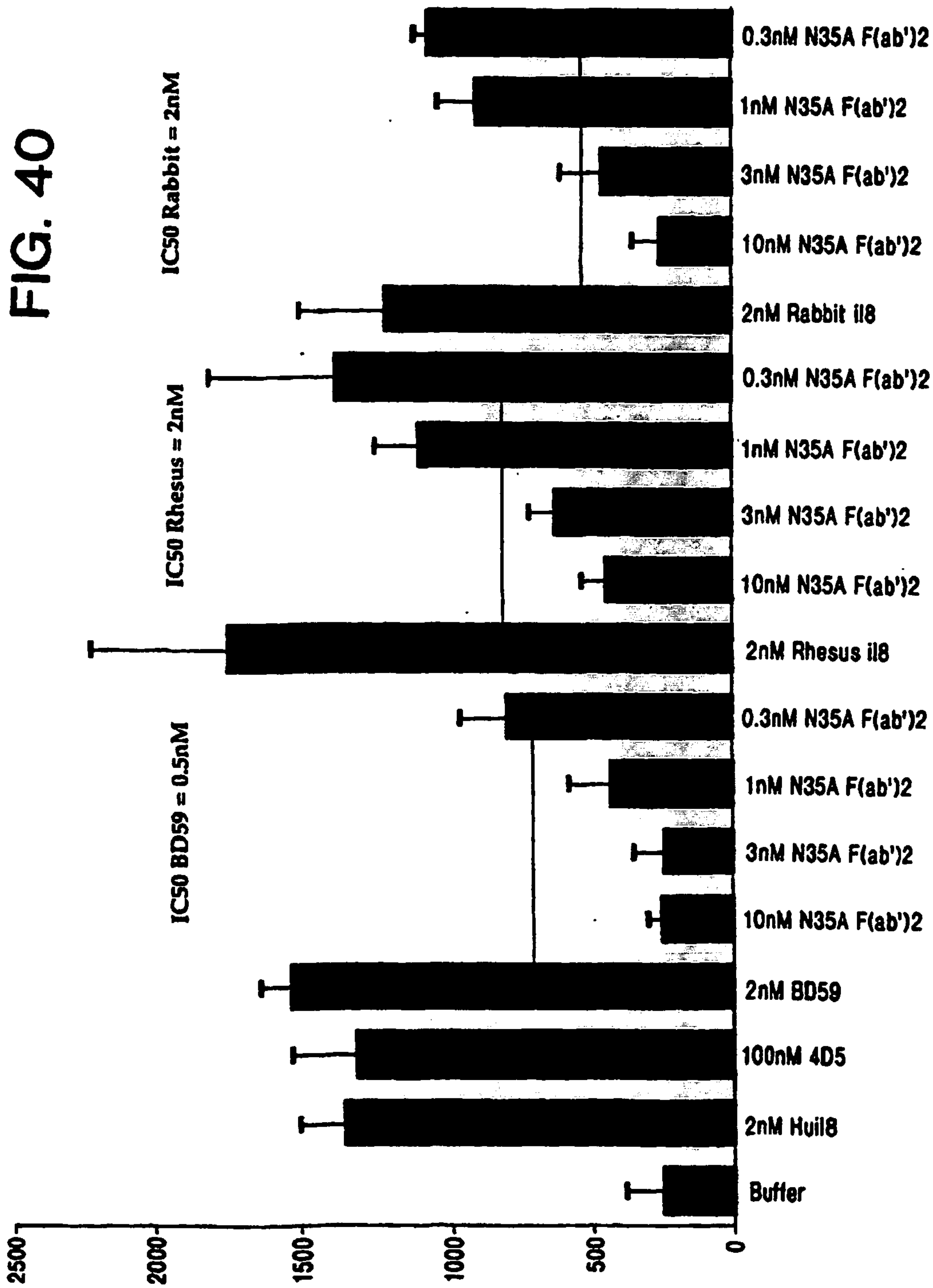

IC50 (F(ab')2) = 0.8nM
IC50 (Fab) = 1.7nM
0.3nM N35A F(ab')2
1nM N35A F(ab')2
3nM N35A F(ab')2
10nM N35A F(ab')2
30nM N35A F(ab')2
100nM N35A F(ab')2
100nM 4D5
2nM HuII8
Buffer
0.3nM V11N35A
1nM N35A Fab
3nM N35A Fab
10nM N35A Fab
30nM N35A Fab
100nM N35A Fab
100nM 4D5
2nM HuII8
Buffer
0
500
1000
1500
2000
2500
3000

FIG. 39

FIG. 40


IC50 Rabbit = 2nM
IC50 Rhesus = 2nM
IC50 BD59 = 0.5nM
0.3nM N35A F(ab')2
1nM N35A F(ab')2
3nM N35A F(ab')2
10nM N35A F(ab')2
2nM Rabbit il8
0.3nM N35A F(ab')2
1nM N35A F(ab')2
3nM N35A F(ab')2
10nM N35A F(ab')2
2nM Rhesus il8
0.3nM N35A F(ab')2
1nM N35A F(ab')2
3nM N35A F(ab')2
10nM N35A F(ab')2
2nM BD59
100nM 4D5
2nM Huil8
Buffer
2500
2000
1500
1000
500
0

```
                                                                                    aluI                   pleI
                                                                                   hindIII             mboII   taqI
      ecoRI         pflMI                                         ddeI         tru9I                  earI/ksp632I
      apoI          bslI                        nlaIII        bsrDI            mseI cac8I          mboII  hinfI
    1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
      CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTTCTTCT TCTCAGCTTA

                  bspMI                                                                              sau3AI
               hinPI                                                                                 mboI/ndeII(dam-)
               hhaI/cfoI                                                                             dpnI(dam+)
              mstI          aluI               mslI                     hinPI             aciI       dpnII(dam-)
              aviII/fspI  hindIII            maeIII bsrDI               hhaI/cfoI      nspBII      bclI(dam-) mnlI
  101 GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
      CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC TGGTTGTCGC CAACTAACTA GTCCATCTCC

                                                                      thaI
                                                                      fnuDII/mvnI
                                                                  fnu4HI
                                                                  bsoFI
                                                                  bbvI        maeII
             rsaI                                              fnu4HI bstUI  snaBI
        hinPI                                                  bsoFI  bsh1236I                 mnlI
        hhaI/cfoI mnlI            cac8I                        bbvI  hinPI   bsaAI                   fokI
       haeII csp6I             sfaNI   bsmI                   aluI   hhaI/cfoI                      sfaNI
  201 GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
      CCCGCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

                                                                                                                aluI
                                                                                                                sstI
                                                                                                                sacI
                                                                                                                hgiJII
                                                haeIII/palI                                                     hgiAI/aspHI
                                               mcrI                                                             ecl136II
                                               eagI/xmaIII/eclXI                                         ecoRI  bsp1286
                                               eaeI                                                  rsaI       bsiHKAI
                          aluI                 cfrI                                                  maeI       bmyI
           tru9I         pvuII                 bsiEI   ahdI/eam1105I                tru9I            bfaI    taqI
           mseI          nspBII            maeIII    bsmAI                          mseI        maeIII  apoI   banII
  301 AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAACTA GAATTCGAGC
      TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CTTAAGCTCG
```

FIG. 41A

```
          scrFI
          nciI
          mspI
          hpaII
          dsaV
         xmaI/pspAI
         smaI
         scrFI
         nciI
         dsaV
         cauII         xhoI
         bsaJI      mnlI
         avaI    sau3AI taqI
      rsaI       mboI/ndeII(dam-)
      csp6I      dpnI(dam+)
     nlaIV      nlaIV  paeR7I
     kpnI cauII dpnII(dam-)
     hgiCI      bstYI/xhoII
     banI bsaJI alwI(dam-)
     asp718     bamHI  avaI        hphI
     acc65I     alwI(dam-) mnlI  mnlI                                   mboII    sfaNI
401  TCGGTACCCG GGGATCCTCT CGAGGTTGAG GTGATTTTAT GAAAAAGAAT ATCGCATTTC TTCTTGCATC TATGTTCGTT TTTTCTATTG CTACAAACGC
     AGCCATGGGC CCCTAGGAGA GCTCCAACTC CACTAAAATA CTTTTTCTTA TAGCGTAAAG AAGAACGTAG ATACAAGCAA AAAAGATAAC GATGTTTGCG
-23                                              M  K  K  N   I  A  F  L   L  A  S   M  F  V   F  S  I  A   T  N  A
       a mutation was found that inactivated the mluI site.  The penultimate nucleotide was changed fr G toT ^

                                      sstI
                                      sacI
                                      hgiJII
                                      hgiAI/aspHI
                                      ecl136II                                          bspMI
                                      bsp1286                                          scfI
                                      bsiHKAI                                          pstI
                                  bsmFI bmyI                              hphI        sse8387I              ddeI   nlaIII
                               bsrI  avaI aluI           mnlI             maeIII     bspMI               aluI   rsaI
            ecoRV          tth111I/aspI  banII        aciI              bstEII  hphI bsgI              hindIII csp6I
501  ATACGCTGAT ATCCAGATGA CCCAGTCCCC GAGCTCCCTG TCCGCCTCTG TGGGCGATAG GGTCACCATC ACCTGCAGGT CAAGTCAAAG CTTAGTACAT
     TATGCGACTA TAGGTCTACT GGGTCAGGGG CTCGAGGGAC AGGCGGAGAC ACCCGCTATC CCAGTGGTAG TGGACGTCCA GTTCAGTTTC GAATCATGTA
-2   Y  A  D  I  Q  M  T   Q  S  P   S  S  L   S  A  S  V   G  D  R   V  T  I   T  C  R  S   S  Q  S   L  V  H
```

FIG. 41B

```
                                              scrFI
                                              mvaI                                            tfiI
                                              ecoRII                                    hinfI       bsmFI
                                              dsaV                                    taqI    bpmI/gsuI[dcm-]
                                              bstNI    aluI                               claI/bsp106   pleI
                            bsrI              apyI[dcm+]                                  bspDI[dam-]   hinfI
601 GGTATAGGTG CTACGTATTT ACACTGGTAT CAACAGAAAC CAGGAAAAGC TCCGAAACTA CTGATTTACA AAGTATCCAA TCGATTCTCT GGAGTCCCTT
    CCATATCCAC GATGCATAAA TGTGACCATA GTTGTCTTTG GTCCTTTTCG AGGCTTTGAT GACTAAATGT TTCATAGGTT AGCTAAGAGA CCTCAGGGAA
32  G  I  G  A   T  Y  L   H  W  Y   Q  Q  K  P   G  K  A   P  K  L   L  I  Y  K   V  S  N   R  F  S   G  V  P  S

                   mspI
                   hpaII
                   bslI
                  bsaWI
                sau3AI
                mboI/ndeII[dam-]
                dpnI[dam+]
                dpnII[dam-]                                     fnu4HI
                alwI[dam-]                                      bsoFI
               nlaIV                                            bbvI
               bstYI/xhoII                                    scfI      mboII                             rsaI
               bamHI                                          pstI      bpuAI                             csp6I
               alwI[dam-]    bsmFI                            bsgI      bbsI                              scaI nlaIII
701 CTCGCTTCTC TGGATCCGGT TCTGGGACGG ATTTCACTCT GACCATCAGC AGTCTGCAGC CAGAAGACTT CGCAACTTAT TACTGTTCAC AGAGTACTCA
    GAGCGAAGAG ACCTAGGCCA AGACCCTGCC TAAAGTGAGA CTGGTAGTCG TCAGACGTCG GTCTTCTGAA GCGTTGAATA ATGACAAGTG TCTCATGAGT
66   R  F  S   G  S  G   S  G  T  D   F  T  L   T  I  S   S  L  Q  P   E  D  F   A  T  Y   Y  C  S  Q   S  T  H

                                styI
                                bsaJI
                             rsaI
                             csp6I
                            nlaIV
                            kpnI
                            hgiCI          sau3AI
        bsrBI               banI           mboI/ndeII[dam-] fnu4HI          mboII
        aciI                asp718         dpnI[dam+]       bsoFI          bpuAI        aciI
     bsmFI     maeII        acc65I         dpnII[dam-]      bbvI           bbsI    mboII
801 TGTCCCGCTC ACGTTTGGAC AGGGTACCAA GGTGGAGATC AAACGAACTG TGGCTGCACC ATCTGTCTTC ATCTTCCCGC CATCTGATGA GCAGTTGAAA
    ACAGGGCGAG TGCAAACCTG TCCCATGGTT CCACCTCTAG TTTGCTTGAC ACCGACGTGG TAGACAGAAG TAGAAGGGCG GTAGACTACT CGTCAACTTT
99  V  P  L   T  F  G  Q   G  T  K   V  E  I   K  R  T  V   A  A  P   S  V  F   I  F  P  P   S  D  E   Q  L  K
```

FIG. 41C

```
                                                                                                              scrFI
                                                                                                              mvaI
                                                                                                              ecoRII
                                                                                                              dsaV
                                                            haeIII/palI                                       bstNI
      xmnI                     xmnI                         haeI       rsaI                     mnlI          bsaJI
      asp700        cac8I asp700                            mnlI       csp6I                    bslI     maeIII apyI[dcm+]
 901 TCTGGAACTG CTTCTGTTGT GTGCCTGCTG AATAACTTCT ATCCCAGAGA GGCCAAAGTA CAGTGGAAGG TGGATAACGC CCTCCAATCG GGTAACTCCC
     AGACCTTGAC GAAGACAACA CACGGACGAC TTATTGAAGA TAGGGTCTCT CCGGTTTCAT GTCACCTTCC ACCTATTGCG GGAGGTTAGC CCATTGAGGG
 132 S  G  T  A   S  V  V   C  L  L   N  N  F  Y   P  R  E   A  K  V   Q  W  K  V   D  N  A   L  Q  S   G  N  S  Q

                                                       fnu4HI
                                                       bsoFI           celII/espI
                                                    ddeI               blpI/bpu1102I
        maeIII                              scfI   mnlI bbvI        hgaI ddeI                                accI  cac8I
1001 AGGAGAGTGT CACAGAGCAG GACAGCAAGG ACAGCACCTA CAGCCTCAGC AGCACCCTGA CGCTGAGCAA AGCAGACTAC GAGAAACACA AAGTCTACGC
     TCCTCTCACA GTGTCTCGTC CTGTCGTTCC TGTCGTGGAT GTCGGAGTCG TCGTGGGACT GCGACTCGTT TCGTCTGATG CTCTTTGTGT TTCAGATGCG
 166  E  S  V   T  E  Q   D  S  K  D   S  T  Y   S  L  S   S  T  L  T   L  S  K   A  D  Y   E  K  H  K   V  Y  A

                        cac8I
                       aluI
                      sstI
                      sacI
                      hgiJII
                      hgiAI/aspHI
                      ecl136II
                      bsp1286
                      bsiHKAI
                      bmyI                                                          mnlI                       rmaI
                  haeIII/palI                                                     sau3AI                       maeI
                 sau96I banII                                                     mboI/ndeII[dam-]             bfaI
                 asuI ddeI                                                   aluI dpnI[dam+]     hgaI        sau96I
        hphI    eco0109I/draII                                             tru9I  dpnII[dam-] mspI           haeIII/palI
       maeIII  alwNI[dcm-]         maeIII     aluI                         mseI   alwI[dam-]  hpaII sfaNI    asuI
1101 CTGCGAAGTC ACCCATCAGG GCCTGAGCTC GCCCGTCACA AAGAGCTTCA ACAGGGGAGA GTGTTAAGCT GATCCTCTAC GCCGGACGCA TCGTGGCCCT
     GACGCTTCAG TGGGTAGTCC CGGACTCGAG CGGGCAGTGT TTCTCGAAGT TGTCCCCTCT CACAATTCGA CTAGGAGATG CGGCCTGCGT AGCACCGGGA
 199  C  E  V   T  H  Q  G   L  S  S   P  V  T   K  S  F  N   R  G  E   C  O
```

FIG. 41D

```
                rmaI                         rmaI
                maeI                         maeI
          rsaI  bfaI                         bfaI        hphI
          csp6I speI                         xbaI mnlI   mnlI                                        mboII    sfaNI
1201 AGTACGCAAC TAGTCGTAAA AAGGGTATCT AGAGGTTGAG GTGATTTTAT GAAAAAGAAT ATCGCATTTC TTCTTGCATC TATGTTCGTT TTTTCTATTG
     TCATGCGTTG ATCAGCATTT TTCCCATAGA TCTCCAACTC CACTAAAATA CTTTTTCTTA TAGCGTAAAG AAGAACGTAG ATACAAGCAA AAAAGATAAC
 -23                                                        M  K  K  N   I  A  F  L   L  A  S   M  F  V   F  S  I  A

                                                                         scrFI
                                                                         mvaI
                                                                         ecoRII
                                                                         dsaV
                  rsaI                                          scrFI
                bsiWI/splI                                      mvaI    fnu4HI
               thaI                                             ecoRII
               fnuDII/mvnI                                      dsaV bstNI hgiJII                         aluI
               bstUI                 rmaI                       bstNI  bsoFI     bsp1286                      alwNI[dcm-]
               bsh1236I              maeI                       apyI[dcm+] bsaJI bmyI                         fnu4HI
              mluI csp6I   mnlI      bfaI                     haeIII/palI  apyI[dcm+]                        bsoFI
              aflIII     ddeI      aluI                 aciI haeI      bbvI      banII                       bbvI
1301 CTACAAACGC GTACGCTGAG GTTCAGCTAG TGCAGTCTGG CGGTGGCCTG GTGCAGCCAG GGGGCTCACT CCGTTTGTCC TGTGCAGCTT CTGGCTACTC
     GATGTTTGCG CATGCGACTC CAAGTCGATC ACGTCAGACC GCCACCGGAC CACGTCGGTC CCCCGAGTGA GGCAAACAGG ACACGTCGAA GACCGATGAG
  -5  T  N  A   Y  A  E   V  Q  L  V   Q  S  G   G  G  L   V  Q  P  G   G  S  L   R  L  S   C  A  A  S   G  Y  S

                                       scrFI
                                       nciI
                                       mspI
                                       hpaII
                                       dsaV
                                       cauII
                                       bslI
                                      xmaI/pspAI
                                      smaI
                                      scrFI
                                      nciI
                                      dsaV        scrFI
                                      cauII       mvaI
                                      bslI        ecoRII
```

FIG. 41E

```
                                        bsaJI       dsaV
                                        avaI        bstNI
          pleI                         bsaJI        bslI                     bslI
          hinfI             sau96I   sau96I         apyI[dcm+]            sau3AI
        taqI                avaII    nlaIV       sau96I              mboI/ndeII[dam-]
       xhoI                 asuI     haeIII/palI asuI                     dpnI[dam+]          maeII
       paeR7I              nlaIV     asuI       eco0109I/draII       dpnII[dam-]    snaBI
       avaI maeIII       bsrI       eco0109I/draII haeIII/palI            alwI[dam-]  hphI    bsaAI
1401 CTTCTCGAGT CACTATATGC ACTGGGTCCG TCAGGCCCCG GGTAAGGGCC TGGAATGGGT TGGATATATT GATCCTTCCA ATGGTGAAAC TACGTATAAT
     GAAGAGCTCA GTGATATACG TGACCCAGGC AGTCCGGGGC CCATTCCCGG ACCTTACCCA ACCTATATAA CTAGGAAGGT TACCACTTTG ATGCATATTA
  29  F  S  S   H  Y  M  H   W  V  R   Q  A  P   G  K  G  L   E  W  V   G  Y  I   D  P  S  N   G  E  T   T  Y  N

                                   thaI
                                   fnuDII/mvnI               scfI
                 haeIII/palI       bstUI                     pstI
                sau96I             bsh1236I                  bsgI             cac8I   mnlI
                asuI             nruI                      bspMI          cac8I   ddeI  drdI
1501 CAAAAGTTCA AGGGCCGTTT CACTTTATCT CGCGACAACT CCAAAAACAC AGCATACCTG CAGATGAACA GCCTGCGTGC TGAGGACACT GCCGTCTATT
     GTTTTCAAGT TCCCGGCAAA GTGAAATAGA GCGCTGTTGA GGTTTTTGTG TCGTATGGAC GTCTACTTGT CGGACGCACG ACTCCTGTGA CGGCAGATAA
  62 Q  K  F  K   G  R  F   T  L  S   R  D  N  S   K  N  T   A  Y  L   Q  M  N  S   L  R  A   E  D  T   A  V  Y  Y

                                                                                                    sau96I
                                                                                                    haeIII/palI
                                                                                                   sau96I
                                                                                                   nlaIV
                                                                                                   hgiJII
                                                                        maeIII                     bsp1286
                                                                       bstEII                      bsp120I
                                                                    scrFI                    bmyI
                                                                    mvaI      mnlI                 banII
                                                 maeII              ecoRII   bsaJI           asuI
                                                ahaII/bsaHI         bstNI     esp3I            styI asuI
                                                hinlI/acyI          dsaV   bseRI             apaI
                                   maeIII     taqI                  bsaJI hphI bsmBI    mnlI  bsaJI
             mnlI               hphI bsrI mboII aatII         nlaIV apyI[dcm+] bsmAI  haeIII/palI eco0109I/draII
1601 ACTGTGCAAG AGGGGATTAT CGCTACAATG GTGACTGGTT CTTCGACGTC TGGGGTCAAG GAACCCTGGT CACCGTCTCC TCGGCCTCCA CCAAGGGCCC
     TGACACGTTC TCCCCTAATA GCGATGTTAC CACTGACCAA GAAGCTGCAG ACCCCAGTTC CTTGGGACCA GTGGCAGAGG AGCCGGAGGT GGTTCCCGGG
  96  C  A  R   G  D  Y   R  Y  N  G   D  W  F   F  D  V   W  G  Q  G   T  L  V   T  V  S   S  A  S  T   K  G  P
                                                               seq right is from p6G425chim2.fab2   ^
```

FIG. 41F

```
                                                                          scrFI
                                                                          mvaI
                                                                scrFI
                                                                mvaI      ecoRII
                     nlaIV                                      ecoRII
                     hgiCI                                      dsaV
                     banI                                       bstNI     ecoNI
                  scrFI                                sau96I dsaV
                  mvaI                                       haeIII/palI  bstNI                         hphI
                  ecoRII                            fnu4HI     fnu4HI                          mspI
                  dsaV          hgiAI/aspHI         bsoFI bsaJI     bslI                       hpaII
          mboII   bstNI     bseRI     bsp1286             aciI  apyI[dcm+]                          cfr10I/bsrFI
         bpuAI    apyI[dcm+]  mnlI    bsiHKAI      bsp1286   asuI    bsoFI                          bsaWI  tth111I/aspI
         bbsI    bsaJI     mnlI       bmyI mnlI    bmyI nspBII bsaJI bbvI apyI[dcm+]          bslI  ageI maeIII
1701 ATCGGTCTTC CCCCTGGCAC CCTCCTCCAA GAGCACCTCT GGGGGCACAG CGGCCCTGGG CTGCCTGGTC AAGGACTACT TCCCCGAACC GGTGACGGTG
     TAGCCAGAAG GGGGACCGTG GGAGGAGGTT CTCGTGGAGA CCCCCGTGTC GCCGGGACCC GACGGACCAG TTCCTGATGA AGGGGCTTGG CCACTGCCAC
 129 S  V  P  P  L  A  P  S  S  K  S  T  S  G  G  T  A  A  L  G  C  L  V  K  D  Y  F  P  E  P  V  T  V

                  hinPI
                  hhaI/cfoI
                  nlaIV               hgiAI/aspHI
                  narI                bsp1286
                  kasI                bsiHKAI     mspI
                  hinlI/acyI       cac8I          hpaII
                  hgiCI     fnu4HI            scrFI              ddeI                 fnu4HI
                  haeII     bsoFI bmyI        nciI              mnlI  pleI            bsoFI   maeIII     mnlI
                  banI      aciI  apaLI/snoI  dsaV              eco81I hinfI      ddeI       hphI   bsp1286
             ddeI ahaII/bsaHI nspBII alw44I/snoI cauII      scfI  bsu36I/mstII/sauI  mnlI bbvI  bstEII bmyI bpmI/gsuI[dcm-]
1801 TCGTGGAACT CAGGCGCCCT GACCAGCGGC GTGCACACCT TCCCGGCTGT CCTACAGTCC TCAGGACTCT ACTCCCTCAG CAGCGTGGTG ACCGTGCCCT
     AGCACCTTGA GTCCGCGGGA CTGGTCGCCG CACGTGTGGA AGGGCCGACA GGATGTCAGG AGTCCTGAGA TGAGGGAGTC GTCGCACCAC TGGCACGGGA
 162 S  W  N  S  G  A  L  T  S  G  V  H  T  F  P  A  V  L  Q  S  S  G  L  Y  S  L  S  S  V  V  T  V  P  S

          aluI        nlaIV
        fnu4HI        hgiCI                                               taqI           hgiJII
        bsoFI         banI                    tfiI                         salI           bsp1286
        bbvI         bsp1286                 hinfI                 styI  hincII/hindII   bmyI
     bstXI           bmyI               maeII                     bsaJI accI             banII          maeIII
1901 CCAGCAGCTT GGGCACCCAG ACCTACATCT GCAACGTGAA TCACAAGCCC AGCAACACCA AGGTCGACAA GAAAGTTGAG CCCAAATCTT GTGACAAAAC
     GGTCGTCGAA CCCGTGGGTC TGGATGTAGA CGTTGCACTT AGTGTTCGGG TCGTTGTGGT TCCAGCTGTT CTTTCAACTC GGGTTTAGAA CACTGTTTTG
 196 S  S  L  G  T  Q  T  Y  I  C  N  V  N  H  K  P  S  N  T  K  V  D  K  K  V  E  P  K  S  C  D  K  T
```

FIG. 41G

```
                                                  fnu4HI
                                                  bsoFI
                                                 haeIII/palI
                                               mcrI
                                               eagI/xmaIII/eclXI
                                               eaeI
                                               cfrI
                                               bsiEI                               sapI
             cac8I                            notI                rmaI             mboII
          nlaIII                              fnu4HI              maeI             earI/ksp632I                 rmaI
         nspI        bsp1286                  bsoFI   nlaIII      bfaI  tth111I/aspI                            maeI
         nspHI  aciI bmyI                     aciI aciI         aluI mnlI       taqI   aluI                     bfaI
2001 TCACACATGC CCGCCGTGCC CAGCACCAGA ACTGCTGGGC GGCCGCATGA AACAGCTAGA GGACAAGGTC GAAGAGCTAC TCTCCAAGAA CTACCACCTA
     AGTGTGTACG GGCGGCACGG GTCGTGGTCT TGACGACCCG CCGGCGTACT TTGTCGATCT CCTGTTCCAG CTTCTCGATG AGAGGTTCTT GATGGTGGAT
229  H  T  C  P  P  C  P  A  P  E  L  L  G  G  R  M  K  Q  L  E  D  K  V  E  E  L  L  S  K  N  Y  H  L
            ^junction between antibody and leucine zipper

                                                                                                  scrFI
                                                      sphI                                        aciI
                                                  ddeI nlaIII                                     mspI
                                                 celII/espI          rmaI                        hpaII
                                                 blpI/bpu1102I       maeI                         dsaV
                                              hinPI   nspI           bfaI      bsmFI              cauII
                                              hhaI/cfoI         sau96I   pleI                  aciI
                      pleI      aluI         haeII    nspHI     haeIII/palI                   fnu4HI
                      hinfI    hindIII       eco47III cac8I     asuI     hinfI                bsoFI
2101 GAGAATGAAG TGGCAAGACT CAAAAAGCTT GTCGGGGAGC GCTAAGCATG CGACGGCCCT AGAGTCCCTA ACGCTCGGTT GCCGCCGGGC GTTTTTTATT
     CTCTTACTTC ACCGTTCTGA GTTTTTCGAA CAGCCCCTCG CGATTCGTAC GCTGCCGGGA TCTCAGGGAT TGCGAGCCAA CGGCGGCCCG CAAAAAATAA
262  E  N  E  V  A  R  L  K  K  L  V  G  E  R  O

      tru9I                             aluI
      mseI                         taqI  hindIII                                             nlaIV
     hpaI   nlaIII              claI/bsp106 tru9I                    tru9I                   hgiCI
     hincII/hindII     aluI     bspDI(dam-) mseI  aciI               mseI                    banI
2201 GTTAACTCAT GTTTGACAGC TTATCATCGA TAAGCTTTAA TGCGGTAGTT TATCACAGTT AAATTGCTAA CGCAGTCAGG CACCGTGTAT GAAATCTAAC
     CAATTGAGTA CAAACTGTCG AATAGTAGCT ATTCGAAATT ACGCCATCAA ATAGTGTCAA TTTAACGATT GCGTCAGTCC GTGGCACATA CTTTAGATTG
```

FIG. 41H

```
                                            sfaNI                                       haeIII/palI
                                        scrFI                                    sau96I
                                        mvaI                                         scrFI
                                        ecoRII                                 nciI
                                        dsaV                            rsaI   mspI mnlI
                            nlaIV       bstNI                                 csp6I  hpaII
                       mnlI hgiCI      bsaJI                           mspI      dsaV bslI
        hinPI          bsaJI       hphI apyI[dcm+]                     hpaII     cauII           ecoRV
        hhaI/cfoI   fokI    banI  maeIII   fokI  scfI                 cfr10I/bsrFI asuI      aciI
2301 AATGCGCTCA TCGTCATCCT CGGCACCGTC ACCCTGGATG CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT
     TTACGCGAGT AGCAGTAGGA GCCGTGGCAG TGGGACCTAC GACATCCGTA TCCGAACCAA TACGGCCATG ACGGCCCGGA GAACGCCCTA TAGCAGGTAA

                                           hinPI
                                           hhaI/cfoI
                                        rmaI
                                        maeI
                                       nheI                                                              fnu4HI
                                   fnu4HI haeII                            hinPI      hgiAI/aspHI        bsoFI
                                   bsoFI  eco47III                         hhaI/cfoI  bsp1286    aciI   haeIII/palI
                     maeIII        bbvI bfaI                              mstI  bslI  bsiHKAI    mcrI      eaeI
           sfaNI  bsrI         cac8I   cac8I               sfaNI          aviII/fspI  bmyI       bsiEI     cfrI
2401 CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT ATATGCGTTG ATGCAATTTC TATGCGCACC CGTTCTCGGA GCACTGTCCG ACCGCTTTGG
     GGCTGTCGTA GCGGTCAGTG ATACCGCACG ACGATCGCGA TATACGCAAC TACGTTAAAG ATACGCGTGG GCAAGAGCCT CGTGACAGGC TGGCGAAACC

                                                                                               mnlI
                                                                                           sau3AI
                                                              sau3AI                       mboI/ndeII[dam-]
                                                              mboI/ndeII[dam-]             dpnI[dam+]
                                                              dpnI[dam+]                   dpnII[dam-]
                                                              dpnII[dam-]                  alwI[dam-]
        aciI                                              thaI                            nlaIV
       fnu4HI                                             fnuDII/mvnI                     bstYI/xhoII      hgaI
       bsoFI                                              bstUI  nlaIII                   bamHI        mspI
     aciI   bsrI     cac8I             nlaIV      taqI    bsh1236I              bslI      alwI[dam-]   hpaII  sfaNI
2501 CCGCCGCCCA GTCCTGCTCG CTTCGCTACT TGGAGCCACT ATCGACTACG CGATCATGGC GACCACACCC GTCCTGTGGA TCCTCTACGC CGGACGCATC
     GGCGGCGGGT CAGGACGAGC GAAGCGATGA ACCTCGGTGA TAGCTGATGC GCTAGTACCG CTGGTGTGGG CAGGACACCT AGGAGATGCG GCCTGCGTAG
```

FIG. 41I

```
                      hinPI
                       hhaI/cfoI
                      nlaIV
                      narI
                      kasI                    hinPI
                      hinlI/acyI               hhaI/cfoI
          mspI        hgiCI                   nlaIV
           hpaII      haeII                   narI
          naeI      mspI                      kasI                                              hgiJII
          cfr10I/bsrFI banI                   hinlI/acyI                                         bsp1286
          cac8I    sgrAI                      hgiCI                                              bmyI
        haeIII/palI hpaII                     haeII                                              banII
        eaeI      hphI ahaII/bsaHI            banI                                           sau3AI cac8I      rcaI  hinPI
        cfrI  sfaNI cfr10I/bsrFI  aciI  cac8I ahaII/bsaHI                                    mboI/ndeII[dam-]  hgiJII  haeII
                                                                                             dpnI[dam+]         bsp1286  eco47III
                                                                      hphI                   dpnII[dam-]        bmyI bspHI hhaI/cfoI
                                                                                         mboII[dam-]            banII nlaIII
2601 GTGGCCGGCA TCACCGGCGC CACAGGTGCG GTTGCTGGCG CCTATATCGC CGACATCACC GATGGGGAAG ATCGGGCTCG CCACTTCGGG CTCATGAGCG
     CACCGGCCGT AGTGGCCGCG GTGTCCACGC CAACGACCGC GGATATAGCG GCTGTAGTGG CTACCCCTTC TAGCCCGAGC GGTGAAGCCC GAGTACTCGC

                                  scrFI
                                  nciI
                                  mspI        hinPI
                                  hpaII        hhaI/cfoI
                            dsaI  dsaV        nlaIV
                            bslI  cauII       narI
                       sau96I  haeIII/palI   kasI
                       nlaIV   eaeI           hinlI/acyI                    fnu4HI
                       haeIII/palI            hgiCI                         bsoFI hgiAI/aspHI
                       asuI bsaJI bsaJI       haeII                         aciI  bsp1286
                      eco0109I/draII          banI                        fnu4HI  bsiHKAI
                    cac8I bslI cfrI  bsmFI    ahaII/bsaHI  cac8I      bslI bsoFI  bmyI
                                                                           aciI  aciI          haeIII/palI
2701 CTTGTTTCGG CGTGGGTATG GTGGCAGGCC CCGTGGCCGG GGGACTGTTG GGCGCCATCT CCTTGCACGC ACCATTCCTT GCGGCGGCGG TGCTCAACGG
     GAACAAAGCC GCACCCATAC CACCGTCCGG GGCACCGGCC CCCTGACAAC CCGCGGTAGA GGAACGTGCG TGGTAAGGAA CGCCGCCGCC ACGAGTTGCC

                                                                                                          mspI
                  fnu4HI                                                                                  hpaII
                  bsoFI  ecoNI    pleI                                                                   bsaWI
     mnlI  bslI  bsrI bbvI  bslI    hinfI     hgaI     sfaNI                     bsrI  aluI bslI
2801 CCTCAACCTA CTACTGGGCT GCTTCCTAAT GCAGGAGTCG CATAAGGGAG AGCGTCGTCC GATGCCCTTG AGAGCCTTCA ACCCAGTCAG CTCCTTCCGG
     GGAGTTGGAT GATGACCCGA CGAAGGATTA CGTCCTCAGC GTATTCCCTC TCGCAGCAGG CTACGGGAAC TCTCGGAAGT TGGGTCAGTC GAGGAAGGCC
```

FIG. 41J

```
                                                                                                  fnu4HI
                                                                                               mspI  hinPI
      aciI                                                                                    naeI  haeII
     thaI                                                                                     cfr10I/bsrFI
     fnuDII/mvnI                                                                              cac8I eco47III
     bstUI   nlaIII                                                                       nlaIV   bsoFI
     bsh1236I              aciI               mboII                                       hgiCI   bbvI
     hinPI  bcgI           fnu4HI          bpuAI                                          banI hpaII hhaI/cfoI
     hhaI/cfoI             bsoFI           bbsI           nlaIII                                                    mnlI
2901 TGGGCGCGGG GCATGACTAT CGTCGCCGCA CTTATGACTG TCTTCTTTAT CATGCAACTC GTAGGACAGG TGCCGGCAGC GCTCTGGGTC ATTTTCGGCG
     ACCCGCGCCC CGTACTGATA GCAGCGGCGT GAATACTGAC AGAAGAAATA GTACGTTGAG CATCCTGTCC ACGGCCGTCG CGAGACCCAG TAAAAGCCGC

                      thaI
                      fnuDII/mvnI
                      bstUI          haeIII/palI                                                          sau96I
      aciI            bsh1236I   sau3AI                                                                   nlaIV
    sau96I           hinPI       mboI/ndeII[dam-]                                                         avaII
    avaII            hhaI/cfoI   dpnI[dam+]           aciI          tfiI                                  asuI
    asuI         bpmI/gsuI[dcm-] dpnII[dam-]        cac8I          hinfI     cac8I mnlI            bsrI    aciI
                                                                                                 maeIII  bsmFI
3001 AGGACCGCTT TCGCTGGAGC GCGACGATGA TCGGCCTGTC GCTTGCGGTA TTCGGAATCT TGCACGCCCT CGCTCAAGCC TTCGTCACTG GTCCCGCCAC
     TCCTGGCGAA AGCGACCTCG CGCTGCTACT AGCCGGACAG CGAACGCCAT AAGCCTTAGA ACGTGCGGGA GCGAGTTCGG AAGCAGTGAC CAGGGCGGTG

                                                  mcrI
                                                  eagI/xmaIII/eclXI
                                                  eaeI    hinPI
                                                  cfrI    hhaI/cfoI                              thaI
                                           mspI   bsiEI  thaI                                   hgaI
                                          naeI   fnu4HI  fnuDII/mvnI                        thaI  fnuDII/mvnI
                                          cfr10I/bsrFI   bstUI                              fnuDII/mvnI
                             haeIII/palI hpaII   bsoFI   bsh1236I                           bstUI bstUI
       maeII                 haeI         cac8I   aciI  hgaI                                bsh1236I mnlI    haeI
      psp1406I            cac8I           bglI nlaIII haeIII/palI      maeII    cac8I      nruI    bsh1236I fokI haeIII/palI
3101 CAAACGTTTC GGCGAGAAGC AGGCCATTAT CGCCGGCATG GCGGCCGACG CGCTGGGCTA CGTCTTGCTG GCGTTCGCGA CGCGAGGCTG GATGGCCTTC
     GTTTGCAAAG CCGCTCTTCG TCCGGTAATA GCGGCCGTAC CGCCGGCTGC GCGACCCGAT GCAGAACGAC CGCAAGCGCT GCGCTCCGAC CTACCGGAAG
```

FIG. 41K

```
                                                                                    bspMI
                                                               thaI              scrFI
                                       fnu4HI                  fnuDII/mvnI       mvaI
                                       bsoFI                   bstUI             ecoRII
                 mboII                 aciI          cac8I           haeI        dsaV
              tfiI                   mspI   mslI   sfaNI   bsh1236I haeIII/palI bstNI
              hinfI                  hpaII sfaNI  fokI   aciI   cac8I  nlaIII   apyI[dcm+]          bsmFI aluI    alwI[dam-]
3201 CCCATTATGA TTCTTCTCGC TTCCGGCGGC ATCGGGATGC CCGCGTTGCA GGCCATGCTG TCCAGGCAGG TAGATGACGA CCATCAGGGA CAGCTTCAAG
     GGGTAATACT AAGAAGAGCG AAGGCCGCCG TAGCCCTACG GGCGCAACGT CCGGTACGAC AGGTCCGTCC ATCTACTGCT GGTAGTCCCT GTCGAAGTTC

              fnu4HI
              bsoFI
              aciI
             thaI                                  sau96I
             fnuDII/mvnI                           avaII
             bstUI                               bsrI       sau3AI                   mnlI
          cac8I                               sau3AI asuI   mboI/ndeII[dam-]         bsaJI   hgiAI/aspHI
     sau3AI bsh1236I                          mboI/ndeII[dam-]                      aciI     bsp1286
     mboI/ndeII[dam-]                         dpnI[dam+] nspBII    maeIII          fnu4HI    bsiHKAI
     dpnI[dam+]                               dpnII[dam-]       dpnI[dam+]         bsoFI     bmyI
     dpnII[dam-]                            taqI[dam-]     aciI dpnII[dam-]        bglI    cac8I  nlaIII       nlaIII
3301 GATCGCTCGC GGCTCTTACC AGCCTAACTT CGATCACTGG ACCGCTGATC GTCACGGCGA TTTATGCCGC CTCGGCGAGC ACATGGAACG GGTTGGCATG
     CTAGCGAGCG CCGAGAATGG TCGGATTGAA GCTAGTGACC TGGCGACTAG CAGTGCCGCT AAATACGGCG GAGCCGCTCG TGTACCTTGC CCAACCGTAC

                fnu4HI
                bsoFI
              hinPI
              hhaI/cfoI                                              haeIII/palI
             nlaIV                                                  sau96I                       fnu4HI
             narI                                                  scrFI                         bsoFI
             kasI                                        thaI      nciI                          aciI
             hinlI/acyI                         thaI     fnuDII/mvnI mspI                       mspI      mnlI
             hgiCI                              fnuDII/mvnI         hpaII                       hpaII nlaIV
             haeII                              bstUI    bstUI      dsaV                       naeI   hgiCI
             banI aciI                          bsh1236I bsh1236I nlaIV asuI   taqI            cfr10I/bsrFI
             ahaII/bsaHI                   mnlI aciI   hgaI aciI   nlaIII cauII  mnlI          cac8I  banI
3401 GATTGTAGGC GCCGCCCTAT ACCTTGTCTG CCTCCCCGCG TTGCGTCGCG GTGCATGGAG CCGGGCCACC TCGACCTGAA TGGAAGCCGG CGGCACCTCG
     CTAACATCCG CGGCGGGATA TGGAACAGAC GGAGGGGCGC AACGCAGCGC CACGTACCTC GGCCCGGTGG AGCTGGACTT ACCTTCGGCC GCCGTGGAGC
```

FIG. 41L

```
                                                                     hinPI                                hgaI
                                                                     hhaI/cfoI                          thaI aciI
              hphI                                                  mstI     pflMI                      fnuDII/mvnI
           tfiI        pflMI                                        aviII/fspI     styI                 bstUI
           hinfI       bslI      nlaIV              aciI         bsmI        bslI  bsaJI                bsh1236I
3501 CTAACGGATT CACCACTCCA AGAATTGGAG CCAATCAATT CTTGCGGAGA ACTGTGAATG CGCAAACCAA CCCTTGGCAG AACATATCCA TCGCGTCCGC
     GATTGCCTAA GTGGTGAGGT TCTTAACCTC GGTTAGTTAA GAACGCCTCT TGACACTTAC GCGTTTGGTT GGGAACCGTC TTGTATAGGT AGCGCAGGCG

                                                        haeIII/palI
                                                       mscI/balI
                                                       haeI
                                                     scrFI
                                                     mvaI dsaI                                              mspI
                                                     ecoRII                                           hpaII
                                                     dsaV                                            scrFI
                                                     bstNI                                                 nciI
                        fnu4HI                       bslI bsaJI              sau3AI                        dsaV
                       thaI hinPI                    apyI[dcm+]              mboI/ndeII[dam-]           sau96I
                 fnu4HI bsoFI                     sau96I              dpnI[dam+]              nlaIV
                 bsoFI fnuDII/mvnI                avaII      hinPI    dpnII[dam-]             avaII            cac8I
             fnu4HI    bstUI                      asuI eaeI  hhaI/cfoI  hgiAI/aspHI           asuI      rmaI
             bsoFI  cac8I   hhaI/cfoI   fnu4HI   ppuMI     mstI nlaIII bsp1286               ppuMI      maeI
             bbvI aciI bsh1236I   avaI  bsoFI    nlaIV cfrI    aviII/fspI  bsiHKAI             eco0109I/draII
        bpmI/gsuI[dcm-] aciI  sfaNI     bbvI     eco0109I/draII  mslI   bmyI              mnlI cauII-bfaI   aciI
3601 CATCTCCAGC AGCCGCACGC GGCGCATCTC GGGCAGCGTT GGGTCCTGGC CACGGGTGCG CATGATCGTG CTCCTGTCGT TGAGGACCCG GCTAGGCTGG
     GTAGAGGTCG TCGGCGTGCG CCGCGTAGAG CCCGTCGCAA CCCAGGACCG GTGCCCACGC GTACTAGCAC GAGGACAGCA ACTCCTGGGC CGATCCGACC

                                                                           fnu4HI
                                                 cac8I                     bsoFI
                                               thaI                        bbvI
                                      hphI     fnuDII/mvnI              fnu4HI
                                  tfiI         bstUI                    bsoFI
                  bsrI            hinfI        bsh1236I  maeII          bbvI        maeII       ddeI        nlaIII
3701 CGGGGTTGCC TTACTGGTTA GCAGAATGAA TCACCGATAC GCGAGCGAAC GTGAAGCGAC TGCTGCTGCA AAACGTCTGC GACCTGAGCA ACAACATGAA
     GCCCCAACGG AATGACCAAT CGTCTTACTT AGTGGCTATG CGCTCGCTTG CACTTCGCTG ACGACGACGT TTTGCAGACG CTGGACTCGT TGTTGTACTT
```

FIG. 41M

```
                                                                                  sau3AI
                                                                                  mboI/ndeII[dam-]
                                                                                  mamI[dam-]
                                                                                  dpnI[dam+]
                                                                                  dpnII[dam-]
                                                                                bstYI/xhoII
                                                                                alwI[dam-]
                                                                              nspI
                                                                              hpaII
                                            aciI                             mroI bsaBI[dam-]          fnu4HI
                                            thaI                             bspMII                    bsoFI
        mboII                               fnuDII/mvnI hinPI                bspEI[dam-]               bbvI
       bpuAI                                bstUI       hhaI/cfoI            bsaWI     sfaNI         sfaNI
       bbsI                                 bsh1236I   haeII       mslI      accIII[dam-]           fokI     cac8I
3801 TGGTCTTCGG TTTCCGTGTT TCGTAAAGTC TGGAAACGCG GAAGTCAGCG CCCTGCACCA TTATGTTCCG GATCTGCATC GCAGGATGCT GCTGGCTACC
     ACCAGAAGCC AAAGGCACAA AGCATTTCAG ACCTTTGCGC CTTCAGTCGC GGGACGTGGT AATACAAGGC CTAGACGTAG CGTCCTACGA CGACCGATGG

                                                                              aciI
                                                                           bsmFI      fokI
                                       cac8I                               sau96I    sfaNI
                                     hinPI                                 nlaIV  aciI
                                     hhaI/cfoI                             avaII  fnu4HI
                             tru9I  haeII                                  asuI  bsoFI              bsrI
                             mseI   eco47III           ddeI                                        aciI            mnlI
3901 CTGTGGAACA CCTACATCTG TATTAACGAA GCGCTGGCAT TGACCCTGAG TGATTTTTCT CTGGTCCCGC CGCATCCATA CCGCCAGTTG TTTACCCTCA
     GACACCTTGT GGATGTAGAC ATAATTGCTT CGCGACCGTA ACTGGGACTC ACTAAAAAGA GACCAGGGCG GCGTAGGTAT GGCGGTCAAC AAATGGGAGT

                     nspI
                 scrFI
                 nciI
                 mspI
         bsrI    hpaII
         bslI    dsaV nlaIII                          mnlI
    maeII        cauII                               fokI
   psp1406I  maeIII  nspHI        maeIII            sfaNI                                  nlaIII    apoI    bslI
4001 CAACGTTCCA GTAACCGGGC ATGTTCATCA TCAGTAACCC GTATCGTGAG CATCCTCTCT CGTTTCATCG GTATCATTAC CCCCATGAAC AGAAATTCCC
     GTTGCAAGGT CATTGGCCCG TACAAGTAGT AGTCATTGGG CATAGCACTC GTAGGAGAGA GCAAAGTAGC CATAGTAATG GGGGTACTTG TCTTTAAGGG
```

FIG. 41N

```
                                                              cac8I
                                                             sau96I
                                                     tru9I   haeIII/palI
                  sfaNI                              mseI    asuI                       tru9I
              mnlI       maeIII               aciI bslI nlaIII aciI                    mseI      bpmI/gsuI[dcm-]
4101 CCTTACACGG AGGCATCAAG TGACCAAACA GGAAAAAACC GCCCTTAACA TGGCCCGCTT TATCAGAAGC CAGACATTAA CGCTTCTGGA GAAACTCAAC
     GGAATGTGCC TCCGTAGTTC ACTGGTTTGT CCTTTTTTGG CGGGAATTGT ACCGGGCGAA ATAGTCTTCG GTCTGTAATT GCGAAGACCT CTTTGAGTTG

                                                                                  fnu4HI   thaI
                                                                                  bsoFI    fnuDII/mvnI
                                                                                aluI       bstUI
                                                                               pvuII      hinPI
              aciI                                                             nspBII     hhaI/cfoI
             thaI                                                             fnu4HI     thaI
             fnuDII/mvnI                 xmnI                                 bsoFI      fnuDII/mvnI
             bstUI                       tfiI                                 bcgI       bstUI
             bsh1236I                    hinfI                                bbvI    mnlI bsh1236I
      aluI hgaI  fokI                    asp700          mslI       aluI    aciI  bbvI   bsh1236I   hphI       hphI
4201 GAGCTGGACG CGGATGAACA GGCAGACATC TGTGAATCGC TTCACGACCA CGCTGATGAG CTTTACCGCA GCTGCCTCGC GCGTTTCGGT GATGACGGTG
     CTCGACCTGC GCCTACTTGT CCGTCTGTAG ACACTTAGCG AAGTGCTGGT GCGACTACTC GAAATGGCGT CGACGGAGCG CGCAAAGCCA CTACTGCCAC

                                 esp3I
                                 bsmBI
                                 bsmAI
                              mspI                                 scrFI                        hgaI
                      fnu4HI  hpaII                                nciI                        thaI
                      bsoFI  scrFI                                 mspI                        fnuDII/mvnI
                      bbvI   nciI                                  hpaII                       bstUI  aciI
                   nlaIII    dsaV                              sfaNI                           bsh1236I
                  nspI       cauII           aluI              fokI dsaV                      hinPI nspBII
         mnlI     nspHI aluI bslI       maeIII              aciI   cauII     drdI             hhaI/cfoI          aciI
4301 AAAACCTCTG ACACATGCAG CTCCCGGAGA CGGTCACAGC TTGTCTGTAA GCGGATGCCG GGAGCAGACA AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG
     TTTTGGAGAC TGTGTACGTC GAGGGCCTCT GCCAGTGTCG AACAGACATT CGCCTACGGC CCTCGTCTGT TCGGGCAGTC CCGCGCAGTC GCCCACAACC
```

FIG. 41O

```
                                                                                                     hgiAI/aspHI
          fnu4HI                                                                                     bsp1286
          bsoFI            maeII                                        sfaNI                        bsiHKAI
          bbvI          maeIII                                        fnu4HI                 ddeI    bmyI  ndeI
        hinPI  nlaIII bsrI bsaAI                    bst1107I  tru9I   bsoFI             rsaI       apaLI/snoI
        hhaI/cfoI  tth111I/aspI           aciI      accI bsrI  mseI   aciI              csp6I      alw44I/snoI
4401 CGGGTGTCGG GGCGCAGCCA TGACCCAGTC ACGTAGCGAT AGCGGAGTGT ATACTGGCTT AACTATGCGG CATCAGAGCA GATTGTACTG AGAGTGCACC
     GCCCACAGCC CCGCGTCGGT ACTGGGTCAG TGCATCGCTA TCGCCTCACA TATGACCGAA TTGATACGCC GTAGTCTCGT CTAACATGAC TCTCACGTGG

                                                              mboII
                                                             earI/ksp632I
                                                            sapI                           hinPI
                                                          hinPI                            hhaI/cfoI
                                                  sfaNI   hhaI/cfoI                     fnu4HI
                                                                                  pleI  bsoFI      mcrI
  aciI           aciI     sfaNI                 aciI    haeII      aciI  mnlI     hinfI bbvI       bsiEI
4501 ATATGCGGTG TGAAATACCG CACAGATGCG TAAGGAGAAA ATACCGCATC AGGCGCTCTT CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG
     TATACGCCAC ACTTTATGGC GTGTCTACGC ATTCCTCTTT TATGGCGTAG TCCGCGAGAA GGCGAAGGAG CGAGTGACTG AGCGACGCGA GCCAGCAAGC

   fnu4HI
   bsoFI
   aciI                                                                        nlaIII         bslI
fnu4HI      aciI                                                              nspI           cac8I
bsoFI   bsrBI                                          tfiI                   nspHI         haeIII/palI
bbvI   cac8I          aluI           aciI              hinfI                  aflIII        haeI
4601 GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA CGGTTATCCA CAGAATCAGG GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA
     CGACGCCGCT CGCCATAGTC GAGTGAGTTT CCGCCATTAT GCCAATAGGT GTCTTAGTCC CCTATTGCGT CCTTTCTTGT ACACTCGTTT TCCGGTCGTT

  scrFI          thaI
  mvaI               fnuDII/mvnI
  ecoRII         bstUI
  dsaV           bsh1236I
  bstNI   bslI      aciI
  apyI[dcm+]       fnu4HI                                                        hgaI
 haeIII/palI       bsoFI    cac8I                   aciI                         drdI
 haeI  nlaIV      haeIII/palI                    nlaIV              sfaNI       taqI              mnlI
4701 AAGGCCAGGA ACCGTAAAAA GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG ACGCTCAAGT CAGAGGTGGC
     TTCCGGTCCT TGGCATTTTT CCGGCGCAAC GACCGCAAAA AGGTATCCGA GGCGGGGGGA CTGCTCGTAG TGTTTTTAGC TGCGAGTTCA GTCTCCACCG
```

FIG. 41P

```
                                             scrFI
                               scrPI   mvaI
                               mvaI          ecoRII
                               ecoRII  dsaV                          bslI
                               dsaV    bstNI            hinPI                aciI    mspI
                               bstNI         apyI[dcm+]  bssSI                   fnu4HI   hpaII
                               apyI[dcm+]   bsaJI   aluI mnlI  hhaI/cfoI         bsoFI    bsaWI        aciI
4801 GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT ACCTGTCCGC
     CTTTGGGCTG TCCTGATATT TCTATGGTCC GCAAAGGGGG ACCTTCGAGG GAGCACGCGA GAGGACAAGG CTGGGACGGC GAATGGCCTA TGGACAGGCG

                                                                                                       hgiAI/aspHI
                                                                                                       bsp1286
                                                                                                       bsiHKAI
                            hinPI                                                                      bmyI
                            hhaI/cfoI                                                                  apaLI/snoI
                           haeII         aluI     scfI       ddeI                          aluI        alw44I/snoI
4901 CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA
     GAAAGAGGGA AGCCCTTCGC ACCGCGAAAG AGTATCGAGT GCGACATCCA TAGAGTCAAG CCACATCCAG CAAGCGAGGT TCGACCCGAC ACACGTGCTT

                                                                                                   alwNI[dcm-]
                        fnu4HI                                          mspI                      fnu4HI
                        bsoFI                                           hpaII                     bsoFI
                      nspBII             maeIII                         scrFI                  fnu4HI
                      aciI  hinPI       mspI                            nciI                   bsoFI
                   mcrI bbvI           bsaWI              pleI          dsaV                   bbvI           maeIII
                   bsiEI    hhaI/cfoI   hpaII             hinfI         cauII              bsrI   bbvI    bsrI
5001 CCCCCCGTTC AGCCCGACCG CTGCGCCTTA TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA
     GGGGGGCAAG TCGGGCTGGC GACGCGGAAT AGGCCATTGA TAGCAGAACT CAGGTTGGGC CATTCTGTGC TGAATAGCGG TGACCGTCGT CGGTGACCAT

                                                                 bslI             rmaI
                                                               haeIII/palI        maeI                      hinPI
                     mnlI        aciI   scfI                  haeI                bfaI                      hhaI/cfoI
5101 ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG ACAGTATTTG GTATCTGCGC
     TGTCCTAATC GTCTCGCTCC ATACATCCGC CACGATGTCT CAAGAACTTC ACCACCGGAT TGATGCCGAT GTGATCTTCC TGTCATAAAC CATAGACGCG
```

FIG. 41Q

```
                                                             mspI
                                                              hpaII
                                                            sau3AI
                                                            mboI/ndeII[dam-]
                                                            dpnI[dam+]
             maeIII                                         dpnII[dam-]                                   fnu4HI
       eco57I bsrI                                 aluI     alwI[dam-]     nspBII                         bsoFI
                                                                           aciI       aciI                bbvI
                                                                                                    cac8I
5201 TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG
     AGACGACTTC GGTCAATGGA AGCCTTTTTC TCAACCATCG AGAACTAGGC CGTTTGTTTG GTGGCGACCA TCGCCACCAA AAAAACAAAC GTTCGTCGTC

                                                    sau3AI
                                             sau3AI mboI/ndeII[dam-]
                                             mboI/ndeII[dam-]
        hinPI           sau3AI   mboII[dam-] dpnI[dam+]
        hhaI/cfoI       mboI/ndeII[dam-]     dpnII[dam-]
       thaI             dpnI[dam+]   dpnI[dam+]
       fnuDII/mvnI      dpnII[dam-]  dpnII[dam-]                                             tru9I        nlaIII
       bstUI           bstYI/xhoII   alwI[dam-]                                              mseI         rcaI
       bsh1236I        alwI[dam-]  bstYI/xhoII                       hgaI ddeI             maeII          bspHI
5301 ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAAGGG ATTTTGGTCA
     TAATGCGCGT CTTTTTTTCC TAGAGTTCTT CTAGGAAACT AGAAAAGATG CCCCAGACTG CGAGTCACCT TGCTTTTGAG TGCAATTCCC TAAAACCAGT

                                   sau3AI
                                   mboI/ndeII[dam-]
                                rmaI
                           hphI    dpnI[dam+]
                      mboII[dam-]
                    sau3AI     maeI        tru9I
                    mboI/ndeII[dam-]       mseI
                    dpnI[dam+]     dpnII[dam-]
                    dpnII[dam-]    alwI[dam-]     tru9I          tru9I
                   bstYI/xhoII   bstYI/xhoII      mseI           mseI
                   alwI[dam-] bfaI       ahaIII/draI           ahaIII/draI                                  maeIII
5401 TGAGATTATC AAAAAGGATC TTCACCTAGA TCCTTTTAAA TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTTA
     ACTCTAATAG TTTTTCCTAG AAGTGGATCT AGGAAAATTT AATTTTTACT TCAAAATTTA GTTAGATTTC ATATATACTC ATTTGAACCA GACTGTCAAT

                      nlaIV             sau3AI
                      hgiCI             mboI/ndeII[dam-]                  pleI
          tru9I       banI              dpnI[dam+]                        hinfI
          mseI        mnlI        ddeI  dpnII[dam-]       fokI            ahdI/eam1105I                     mnlI
5501 CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG
     GGTTACGAAT TAGTCACTCC GTGGATAGAG TCGCTAGACA GATAAAGCAA GTAGGTATCA ACGGACTGAG GGGCAGCACA TCTATTGATG CTATGCCCTC
```

FIG. 41R

```
                                             bsmAI
                                             bsaI
                      bsrI                thaI                   bpmI/gsuI[dcm-]
                  sau96I    fnu4HI        fnuDII/mvnI        mspI                                 mspI
                  nlaIV     bsoFI         bstUI              hpaII                                hpaII   haeIII/palI
                  haeIII/palI  bsrDI      bsh1236I          cfr10I/bsrFI                         bglI    sau96I hinPI
                  asuI      bbvI         aciI           hphI  nlaIV                         cac8I       asuI   hhaI/cfoI
5601 GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA GCCAGCCGGA AGGGCCGAGC
     CCGAATGGTA GACCGGGGTC ACGACGTTAC TATGGCGCTC TGGGTGCGAG TGGCCGAGGT CTAAATAGTC GTTATTTGGT CGGTCGGCCT TCCCGGCTCG

                                                              scrFI
                                                              nciI
                                                              mspI                                           maeII
                                                              hpaII    rmaI                          hinPI
             sau96I                                 tru9I     dsaV     maeI                          hhaI/cfoI
             avaII           mnlI    bsrI     mseI       cauII    bfaI                   tru9I     mstI psp1406I
             asuI             aciI    fokI       aseI/asnI/vspI    aluI                 bsrI mseI      aviII/fspI
5701 GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT
     CGTCTTCACC AGGACGTTGA AATAGGCGGA GGTAGGTCAG ATAATTAACA ACGGCCCTTC GATCTCATTC ATCAAGCGGT CAATTATCAA ACGCGTTGCA

                   cac8I
                 scfI                                                                                    sau3AI
                 pstI                                                                                    mboI/ndeII[dam-]
                fnu4HI                                                     nlaIV      sau3AI              dpnI[dam+]
                bsoFI                                                    mspI         mboI/ndeII[dam-]   dpnII[dam-]
                bbvI    mslI                                            bsaWI         dpnI[dam+]    nlaIII     nlaIII
           bsrDI bsgI  sfaNI      maeIII                            aluI hpaII        dpnII[dam-] maeIII  alwI[dam-]
5801 TGTTGCCATT GCTGCAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC
     ACAACGGTAA CGACGTCCGT AGCACCACAG TGCGAGCAGC AAACCATACC GAAGTAAGTC GAGGCCAAGG GTTGCTAGTT CCGCTCAATG TACTAGGGGG

                                              sau3AI
                                              mboI/ndeII[dam-]         aciI
                                              dpnI[dam+]              fnu4HI
                                         mnlI dpnII[dam-]             bsoFI
                                      sau96I pvuI/bspCI            haeIII/palI                  fnu4HI
                                      avaII  mcrI                 eaeI             nlaIII      bsoFI
                     aciI   aluI      asuI   bsiEI                cfrI             mslI        bbvI
5901 ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTTATGGCA GCACTGCATA
     TACAACACGT TTTTTCGCCA ATCGAGGAAG CCAGGAGGCT AGCAACAGTC TTCATTCAAC CGGCGTCACA ATAGTGAGTA CCAATACCGT CGTGACGTAT
```

FIG. 41S

```
                                                                                                mcrI
                                                                                                 bsiEI
                                                                                                 bcgI
                                                            rsaI                             fnu4HI
          fokI                                    bsrI      scaI                             bsoPI
      nlaIII              sfaNI             maeIII hphI   csp6I            ddeI              aciI
6001 ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC
     TAAGAGAATG ACAGTACGGT AGGCATTCTA CGAAAAGACA CTGACCACTC ATGAGTTGGT TCAGTAAGAC TCTTATCACA TACGCCGCTG GCTCAACGAG

        hgaI
       hinlI/acyI
       ahaII/bsaHI              hinPI
      mspI                       hhaI/cfoI                                                                    sau3AI
      hpaII                     thaI                          hgiAI/aspHI                                     mboI/ndeII[dam-]
     scrFI                      fnuDII/mvnI                   bsp1286        maeII                            dpnI[dam+]
     nciI                       bstUI                 tru9I   bsiHKAI       psp1406I                          dpnII[dam-]
     dsaV                       bsh1236I              mseI    bmyI        xmnI                               bstYI/xhoII
     cauII hincII/hindII       aciI                 ahaIII/draI           asp700    mboII                    alwI[dam-]
6101 TTGCCCGGCG TCAACACGGG ATAATACCGC GCCACATAGC AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG GGCGAAAACT CTCAAGGATC
     AACGGGCCGC AGTTGTGCCC TATTATGGCG CGGTGTATCG TCTTGAAATT TTCACGAGTA GTAACCTTTT GCAAGAAGCC CCGCTTTTGA GAGTTCCTAG

                bsrI                     hgiAI/aspHI
              sau3AI    taqI             bsp1286          eco57I
              mboI/ndeII[dam-]           bsiHKAI          mboII[dam-]
              dpnI[dam+]                 bmyI           sau3AI   sfaNI
              dpnII[dam-]                apaLI/snoI     mboI/ndeII[dam-]
     nspBII   alwI[dam-]                 alw44I/snoI    dpnI[dam+]
     aciI    bstYI/xhoII       maeIII  bssSI            dpnII[dam-]          hphI          hphI
6201 TTACCGCTGT TGAGATCCAG TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT TTCTGGGTGA GCAAAAACAG
     AATGGCGACA ACTCTAGGTC AAGCTACATT GGGTGAGCAC GTGGGTTGAC TAGAAGTCGT AGAAAATGAA AGTGGTCGCA AAGACCCACT CGTTTTTGTC

              aciI
             fnu4HI                                            mboII
             bsoPI                    mslI                   earI/ksp632I  sspI
6301 GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA
     CTTCCGTTTT ACGGCGTTTT TTCCCTTATT CCCGCTGTGC CTTTACAACT TATGAGTATG AGAAGGAAAA AGTTATAATA ACTTCGTAAA TAGTCCCAAT
```

FIG. 41T

```
                                                                                      hinPI
                                                                                    thaI
                                                                                    fnuDII/mvnI
         nlaIII                                                                     bstUI                                      maeII
        rcaI                                                                        bsh1236I                                  hinlI/acyI
        bspHI  aciI                                                                aciI                                       ahaII/bsaHI
      bsmAI  bsrBI                                                             nlaIV   hhaI/cfoI                              aatII ddeI
6401 TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC GCGCACATTT CCCCGAAAAG TGCCACCTGA CGTCTAAGAA
     AACAGAGTAC TCGCCTATGT ATAAACTTAC ATAAATCTTT TTATTTGTTT ATCCCCAAGG CGCGTGTAAA GGGGCTTTTC ACGGTGGACT GCAGATTCTT

                                                                  sau96I
                                                                  haeIII/palI
                                                                  asuI        mboII
            nlaIII                                               ecoO109I/draII
           rcaI     tru9I                                       mnlI        bpuAI
           bspHI    mseI                                     bssSI          bbsI
6501 ACCATTATTA TCATGACATT AACCTATAAA AATAGGCGTA TCACGAGGCC CTTTCGTCTT CAA
     TGGTAATAAT AGTACTGTAA TTGGATATTT TTATCCGCAT AGTGCTCCGG GAAAGCAGAA GTT
```

FIG. 41U

>length: 6563

aatII(GACGTC):	1645 6489
acc65I(GGTACC):	403 823
accI(GTMKAC):	1093 1963 4449
accIII(TCCGGA):	3867[dam-]
aciI(CCGC):	178 542 805 877 1340 1750 1826 2011 2039 2043 2182 2242 2384 2492 2501 2504 2628 2781 2784 2787 2906 2926 3005 3045 3094 3141 3226 3241 3309 3342 3367 3412 3436 3448 3490 3544 3597 3613 3619 3700 3838 3967 3970 3981 4139 4155 4210 4266 4351 4390 4400 4442 4467 4505 4518 4544 4561 4604 4611 4632 4723 4751 4878 4897 5018 5128 5263 5272 5634 5725 5916 5962 6083 6127 6204 6313 6412 6459
acyI	see hinlI
aflIII(ACRYGT):	1307 4678
ageI(ACCGGT):	1788
ahaII/bsaHI(GRCGYC):	1645 1813 2616 2637 2751 3408 6107 6489
ahaIII/draI(TTTAAA):	5435 5454 6146
ahdI/eam1105I(GACNNNNNGTC):	346 5566
aluI(AGCT):	72 121 252 320 398 532 589 648 1126 1144 1167 1325 1386 1906 2054 2075 2126 2218 2233 2889 3292 4202 4259 4270 4319 4338 4619 4845 4935 4981 5238 5759 5859 5922
alw44I/snoI(GTGCAC):	1831 4494 4992 6238
alwI[dam-](GGATC):	412 413 712 713 1171 1471 2578 2579 3300 3870 5245 5319 5331 5416 5429 5893 6196 6214
alwNI[dcm-](CAGNNNCTG):	1117 1385 5089
apaI(GGGCCC):	1695
apaLI/snoI(GTGCAC):	1831 4494 4992 6238
apoI(RAATTY):	1 391 4093
apyI[dcm+](CCWGG):	640 999 1347 1357 1449 1665 1713 1755 1764 2333 3262 3645 4705 4826 4839
aseI/asnI/vspI(ATTAAT):	5742
asnI	see aseI
asp700(GAANNNNTTC):	905 930 4234 6166
asp718(GGTACC):	403 823
aspHI	see hgiAI
aspI	see tth111I
asuI(GGNCC):	1119 1195 1425 1434 1446 1512 1695 1696 1752 2155 2375 2727 3002 3090 3339 3463

FIG. 41V

Stop Template Primer

SL.97.2 5' CAT GGT ATA GGT <u>TAA</u> ACT TAT TTA CAC 3'

NNS Randomization Primer

SL.97.3 5' CAT GGT ATA GGT <u>NNS</u> ACT TAT TTA CAC 3'

FIG. 42

Randomization of Position N35 of Variable Light Chain CDR-1
Amino Acid Frequency

Phage Display (NNS Codon Library) Sort #3

Amino Acid	Frequency	% Total	IC50 (nM)
Asparagine (wt)	1	5.6	4.9
Glycine	6	16.6	3.1
Aspartic Acid	3	16.6	3.1
Glutamic Acid	4	22.2	0.1
Alanine	2	5.6	0.2
Lysine	1	5.6	ND
Serine	1	1.9	ND

FIG. 43A

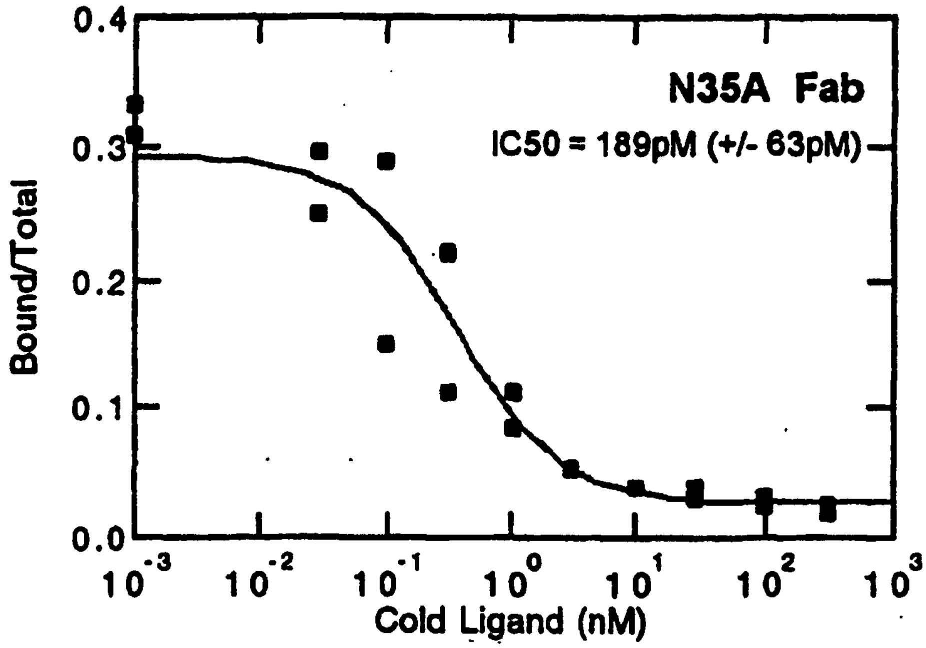


FIG. 43B-1

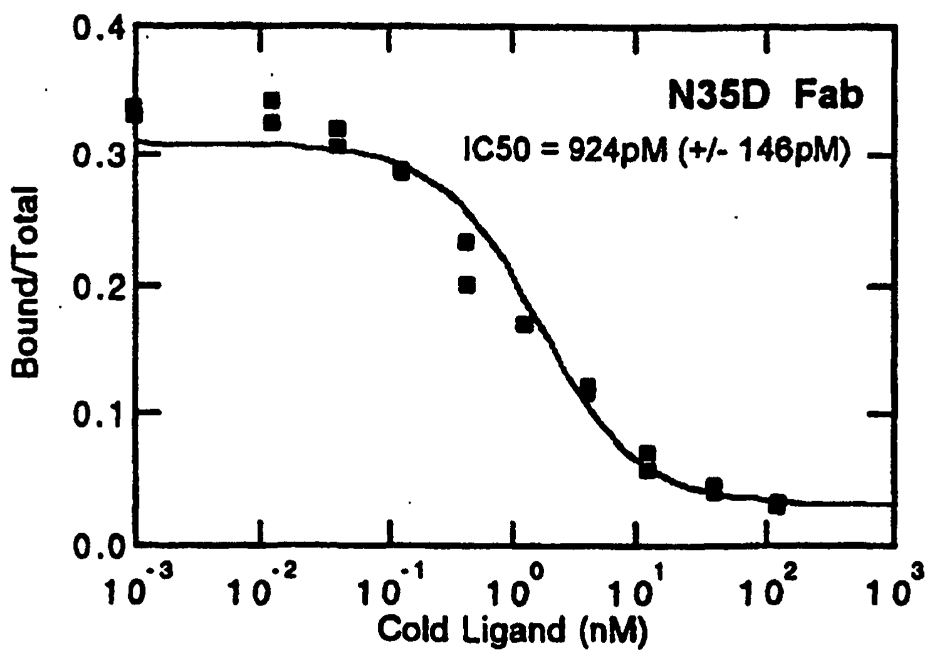


FIG. 43B-2

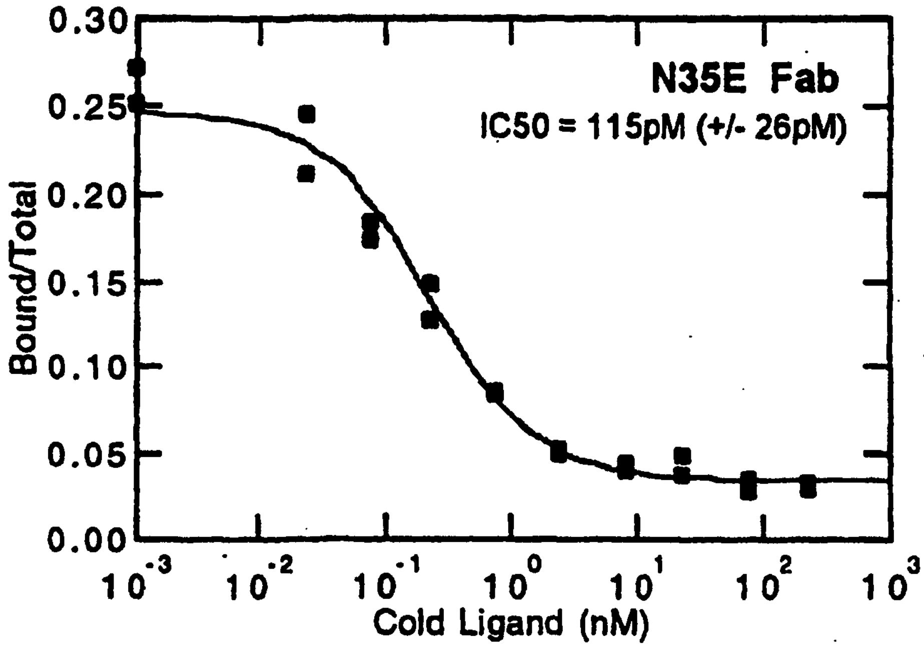


FIG. 43B-3

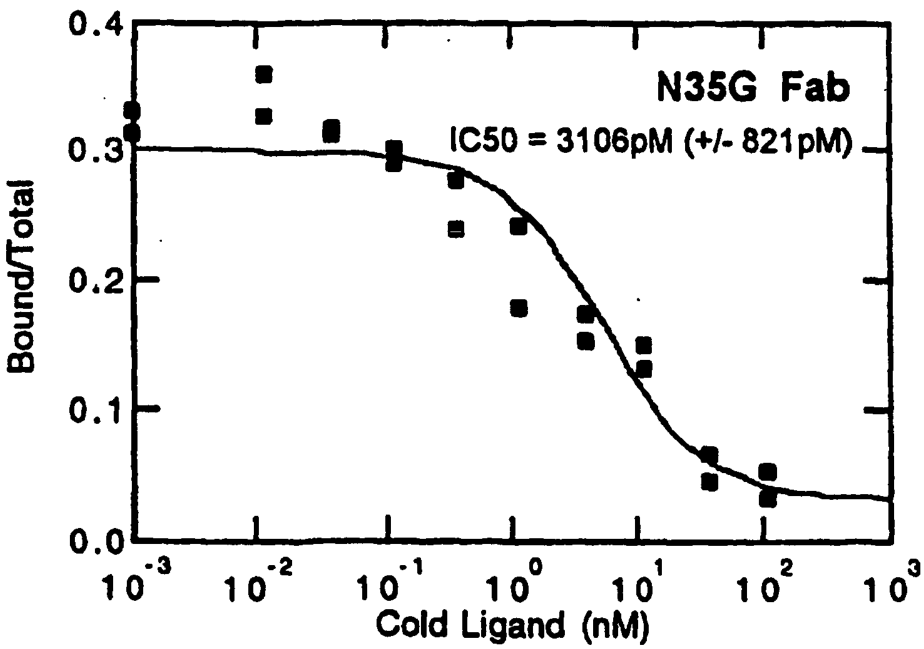
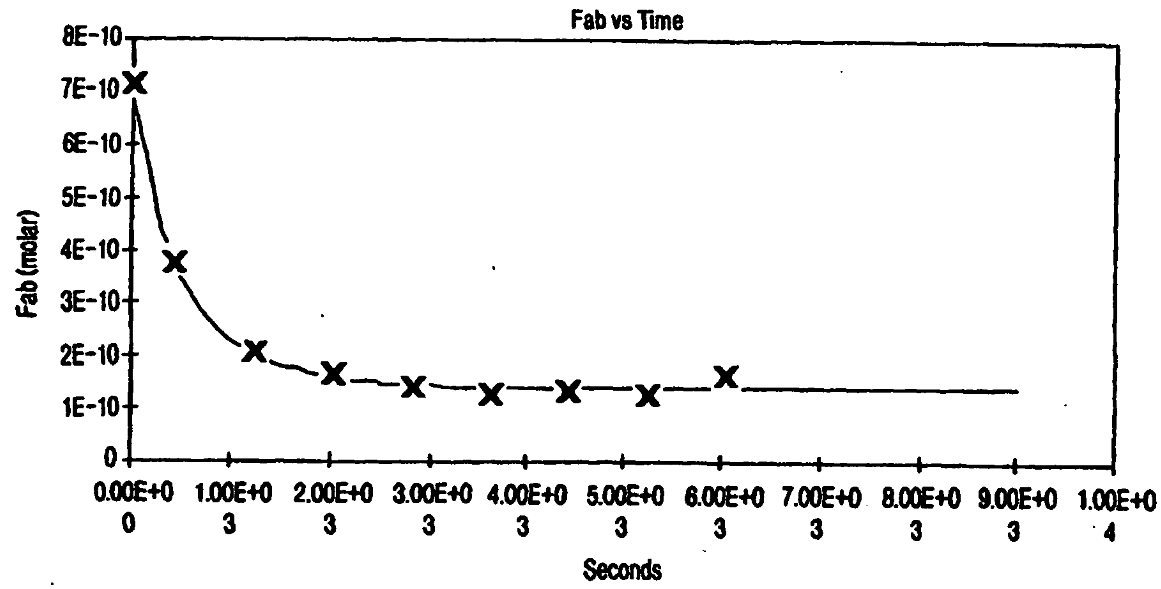


FIG. 43B-4



Representative Conc versus Time Plot. Shown is the kinetic data for 6G4V11N35A.F(ab')2.

SAMPLE	ka	kd	Kd
6G4V11N35A-Fab	ND	ND	114pM
6G4V11N35A-F(ab')$_2$	$2.0x10^6$	$2.1x10^{-4}$	109pM
6G4V11N35E-Fab	$4.7x10^6$	$2.6x10^{-4}$	54pM

FIG. 44

```
  1 ATGAAAAAGA ATATCGCATT TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC
    TACTTTTTCT TATAGCGTAA AGAAGAACGT AGATACAAGC AAAAAAGATA ACGATGTTTG
-23 M  K  K  N   I  A  F   L  L  A   S  M  F  V   F  S  I   A  T  N

 61 GCATACGCTG ATATCCAGAT GACCCAGTCC CCGAGCTCCC TGTCCGCCTC TGTGGGCGAT
    CGTATGCGAC TATAGGTCTA CTGGGTCAGG GGCTCGAGGG ACAGGCGGAG ACACCCGCTA
 -3 A  Y  A  D   I  Q  M   T  Q  S   P  S  S  L   S  A  S   V  G  D

121 AGGGTCACCA TCACCTGCAG GTCAAGTCAA AGCTTAGTAC ATGGTATAGG TGAGACGTAT
    TCCCAGTGGT AGTGGACGTC CAGTTCAGTT TCGAATCATG TACCATATCC ACTCTGCATA
 18 R  V  T  I   T  C  R   S  S  Q   S  L  V  H   G  I  G   E  T  Y

181 TTACACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA CAAAGTATCC
    AATGTGACCA TAGTTGTCTT TGGTCCTTTT CGAGGCTTTG ATGACTAAAT GTTTCATAGG
 38 L  H  W  Y   Q  Q  K   P  G  K   A  P  K  L   L  I  Y   K  V  S

241 AATCGATTCT CTGGAGTCCC TTCTCGCTTC TCTGGATCCG GTTCTGGGAC GGATTTCACT
    TTAGCTAAGA GACCTCAGGG AAGAGCGAAG AGACCTAGGC CAAGACCCTG CCTAAAGTGA
 58 N  R  F  S   G  V  P   S  R  F   S  G  S  G   S  G  T   D  F  T

301 CTGACCATCA GCAGTCTGCA GCCAGAAGAC TTCGCAACTT ATTACTGTTC ACAGAGTACT
    GACTGGTAGT CGTCAGACGT CGGTCTTCTG AAGCGTTGAA TAATGACAAG TGTCTCATGA
 78 L  T  I  S   S  L  Q   P  E  D   F  A  T  Y   Y  C  S   Q  S  T

361 CATGTCCCGC TCACGTTTGG ACAGGGTACC AAGGTGGAGA TCAAACGAAC TGTGGCTGCA
    GTACAGGGCG AGTGCAAACC TGTCCCATGG TTCCACCTCT AGTTTGCTTG ACACCGACGT
 98 H  V  P  L   T  F  G   Q  G  T   K  V  E  I   K  R  T   V  A  A

421 CCATCTGTCT TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCTTCTGTT
    GGTAGACAGA AGTAGAAGGG CGGTAGACTA CTCGTCAACT TTAGACCTTG ACGAAGACAA
118 P  S  V  F   I  F  P   P  S  D   E  Q  L  K   S  G  T   A  S  V

481 GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA GGTGGATAAC
    CACACGGACG ACTTATTGAA GATAGGGTCT CTCCGGTTTC ATGTCACCTT CCACCTATTG
138 V  C  L  L   N  N  F   Y  P  R   E  A  K  V   Q  W  K   V  D  N

541 GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC AGGACAGCAA GGACAGCACC
    CGGGAGGTTA GCCCATTGAG GGTCCTCTCA CAGTGTCTCG TCCTGTCGTT CCTGTCGTGG
158 A  L  Q  S   G  N  S   Q  E  S   V  T  E  Q   D  S  K   D  S  T

601 TACAGCCTCA GCAGCACCCT GACGCTGAGC AAAGCAGACT ACGAGAAACA CAAAGTCTAC
    ATGTCGGAGT CGTCGTGGGA CTGCGACTCG TTTCGTCTGA TGCTCTTTGT GTTTCAGATG
178 Y  S  L  S   S  T  L   T  L  S   K  A  D  Y   E  K  H   K  V  Y

661 GCCTGCGAAG TCACCCATCA GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA
    CGGACGCTTC AGTGGGTAGT CCCGGACTCG AGCGGGCAGT GTTTCTCGAA GTTGTCCCCT
198 A  C  E  V   T  H  Q   G  L  S   S  P  V  T   K  S  F   N  R  G

721 GAGTGTTAAG CTGATCCTCT ACGCCGGACG CATCGTGGCC CTAGTACGCA ACTAGTCGTA
    CTCACAATTC GACTAGGAGA TGCGGCCTGC GTAGCACCGG GATCATGCGT TGATCAGCAT
218 E  C  O
```

FIG. 45

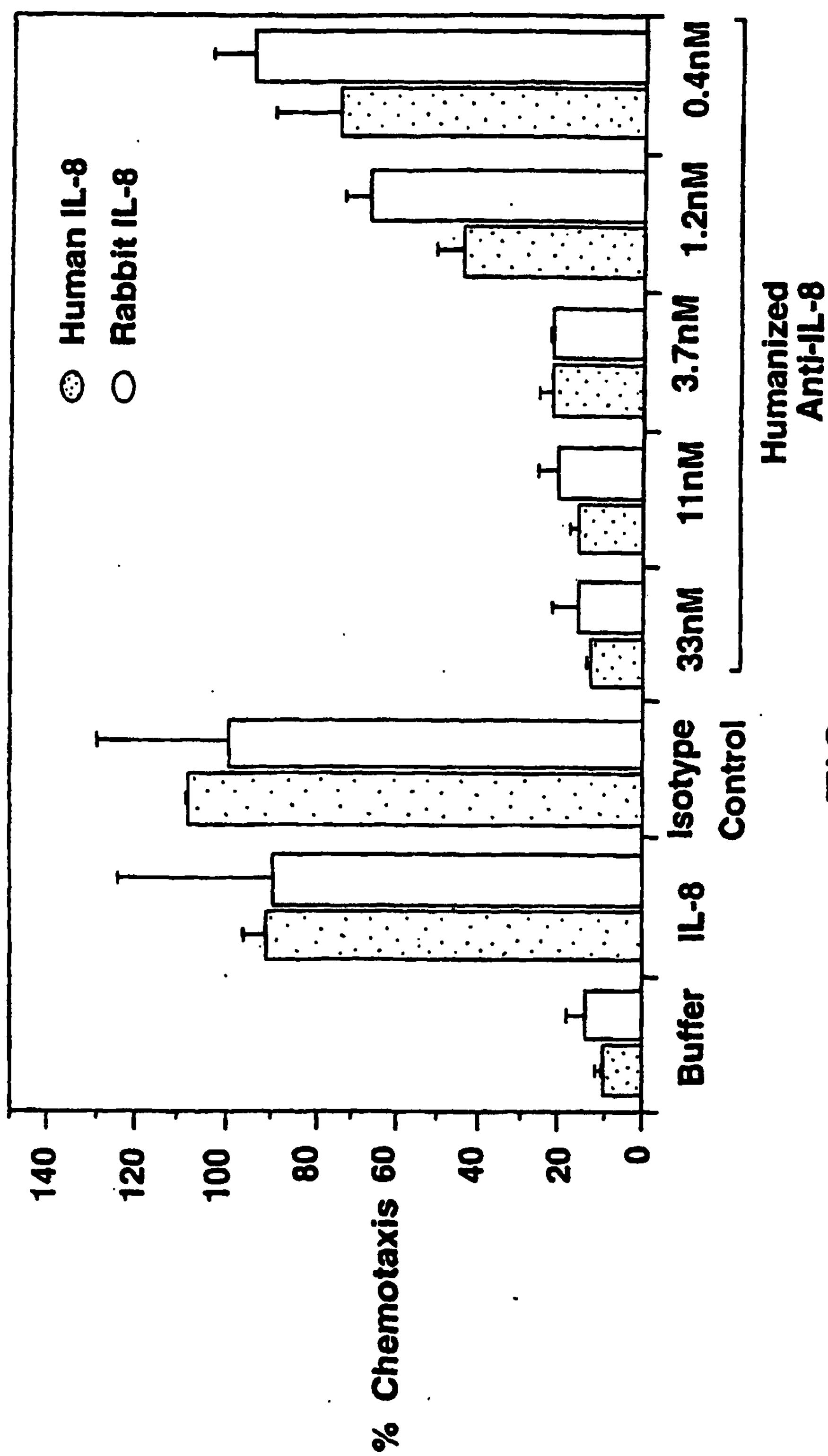

Human IL-8
Rabbit IL-8
% Chemotaxis
140
120
100
80
60
40
20
0
Buffer
IL-8
Isotype Control
33nM
11nM
3.7nM
1.2nM
0.4nM
Humanized Anti-IL-8

FIG. 46

N35AH1upr

5'-CTAGTGCAGTCTGGCGGTGGCCTGGTGCAGCCAGGGGGCTCACTCCGTTTGTCCTGTGCAGCTTCTGGCTACTCCTTC-3'

N35AH1lwr

5'-TCGAGAAGGAGTAGCCAGAAGCTGCACAGGACAAACGGAGTGAGCCCCCTGGCTGCACCAGGCCACCGCCAGACTGCACT
AG-3'

Bold indicates nucleotide change destroying PvuII site.

FIG. 47

```
> Wed May  7 18:27:36 1997
> /home/ruby/vc/Immbio/afan/ss.p6G425v11.N35A.choSD
> sites: std
> length: 8120 (circular)
>This has the pSVI backbone with the pRK7 cloning linker (pSVI7) and the intron DHFR(ID)
>made from pSVI.WTSD.D by adding a linearization linker(LL) into the Hpal site

         cac8I
        aluI
       sstI
       sacI                                    sau3AI  aluI
       hgiJII                                  mboI/ndeII[dam-]                               scrFI
       hgiAI/aspHI                             dpnI[dam+]                                     mvaI
       ecl136II                               pvuI/bspCI                                      ecoRII
       bsp1286                             pleI dpnII[dam-]                                   dsaV
       bsiHKAI                             hinfI  taqI[dam-]                                  bstNI
       bmyI                            rmaI    mcrI    pvuII                                  apyI[dcm+]
       banII                           maeI    bsiEI   nspBII                                bsaJI
     taqI                              bfaI   taqI[dam-]                                  bsmFI   nlaIV     cac8I
   1 TTCGAGCTCG CCCGACATTG ATTATTGACT AGAGTCGATC GACAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA
     AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCTCAGCTAG CTGTCGACAC CTTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG GGTCGTCCGT

                sfaNI                                       scrFI                               sfaNI
              ppu10I                   scrFI       mvaI                                       ppu10I
              nsiI/avaIII              mvaI               ecoRII                              nsiI/avaIII
             nlaIII                    ecoRII      dsaV                                  nlaIII
            sphI                       dsaV        bstNI                                      sphI
            nspI                       bstNI          apyI[dcm+]                                    nspI
            nspHI                      apyI[dcm+]    bsaJI                                          nspHI
            cac8I                     sexAI        bsmFI   nlaIV      cac8I                         cac8I
 101 GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
     CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC AGGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG TAGAGTTAAT

                                                                                   nlaIII
                                                                                  styI
                                                                                  ncoI
                aciI                       aciI                               bslI dsaI
              bsmFI             aciI   fokI                aciI bsrI   aciI     aciI bsaJI
 201 GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT TTTTTTTATT
     CAGTCGTTGG TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC CGACTGATTA AAAAAAATAA
```

FIG. 48A

```
                                                                                          rmaI
                 fnu4HI                                                                   maeI
                 bsoFI                                                                   styI                         haeIII/palI
                 bglI                                                                    bsaJI               aluI    mcrI
                sfiI                                                                     blnI               rmaI    eagI/xmaIII/eclXI
                haeIII/palI                                                              avrII(dam-)        maeI    eaeI
              mnlI   mnlI      ddeI                                                   haeIII/palI           bfaI    cfrI
          haeIII/palI bsaJI mnlI   aluI                          mnlI                    stuI                nheI    bsiEI
          mnlI bsaJI aciI   haeIII/palI                     mnlI                         haeI                cac8I   mspI
                                                            bseRI                       mnlI bfaI            aluI    hpaII
301 TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTA GCTTATCCGG
    ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC TCCTCCGAAA AAACCTCCGG ATCCGAAAAC GTTTTTCGAT CGAATAGGCC

                        tfiI
    scrFI               hinfI
    nciI               aciI                                                              fnu4HI
    mspI               thaI                                                              bsoFI
    hpaII              fnuDII/mvnI                       aciI                            bbvI
    dsaV               bstUI                    maeII   rsaI                             nspBII
    cauII              bsh1236I                maeIII   csp6I  scfI        mnlI          aciI       nlaIII
401 CCGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTGACGTAA GTACCGCCTA TAGAGCGATA AGAGGATTTT ATCCCCGCTG CCATCATGGT
    GGCCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCACTGCATT CATGGCGGAT ATCTCGCTAT TCTCCTAAAA TAGGGGCGAC GGTAGTACCA
                                                 ^splice donor                                   DHFR ATG^

                                                                          haeIII/palI
                                                                         haeI
                                                                        scrFI
                                                                        mvaI     bsrBI
                                                                        ecoRII
                                                                        dsaV
                                   pflMI                                bstNI    aciI                 rsaI
                                   bslI                         bsmAI   apyI(dcm+)         xmnI       csp6I
    taqI        sfaNI           bsmFI                           bsaI   bsaJI  mnlI  ddeI   asp700     scaI
501 TCGACCATTG AACTGCATCG TCGCCGTGTC CCAAAATATG GGGATTGGCA AGAACGGAGA CCTACCCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC
    AGCTGGTAAC TTGACGTAGC AGCGGCACAG GGTTTTATAC CCCTAACCGT TCTTGCCTCT GGATGGGACC GGAGGCGAGT CCTTGCTCAA GTTCATGAAG
```

FIG. 48B

```
                                                                          scrFI
                                                                          mvaI
                                                                          ecoRII
                        eco57I                                            dsaV
                       mboII                     tfiI                     bstNI                   tfiI       tru9I
                      earI/ksp632I               hinfI  hphI              apyI[dcm+]              hinfI      mseI
                     mnlI                      alwNI[dcm-]               sexAI          ddeI mboII taqI     ahaIII/draI
601 CAAAGAATGA CCACAACCTC TTCAGTGGAA GGTAAACAGA ATCTGGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC TGAGAAGAAT CGACCTTTAA
    GTTTCTTACT GGTGTTGGAG AAGTCACCTT CCATTTGTCT TAGACCACTA ATACCCATCC TTTTGGACCA AGAGGTAAGG ACTCTTCTTA GCTGGAAATT

                                                            sstI
                                                            sacI
                                                            hgiJII
                                                            hgiAI/aspHI
                                                            ecl136II
                                                            bsp1286
                                                            bsiHKAI
                                                            bmyI
         tru9I                                          mnlI aluI                                 tru9I
         mseI         ddeI                           bssSI  banII                                aflII/bfrI
        aseI/asnI/vspI                           belI   bseRI               bstXI      fokI sfaNI mseI
701 AGGACAGAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA ACCACCACGA GGAGCTCATT TTCTTGCCAA AAGTTTGGAT GATGCCTTAA GACTTATTGA
    TCCTGTCTTA ATTATATCAA GAGTCATCTC TTGAGTTTCT TGGTGGTGCT CCTCGAGTAA AAGAACGGTT TTCAAACCTA CTACGGAATT CTGAATAACT

                                                                                             naeIII/palI
                                                                                            haeI
                                                                      scrFI         scrFI
                                                                      mvaI                mvaI
                                                                      ecoRII        ecoRII
                                                                      dsaV   tfiI   dsaV
    mspI                                                              bstNI    nlaIII     bstNI     ddeI pleI
    hpaII                                                             apyI[dcm+]   hinfI  apyI[dcm+]     hinfI
   bsaWI                    accI nlaIII          mnlI
801 ACAACCGGAA TTGGCAAGTA AAGTAGACAT GGTTTGGATA GTCGGAGGCA GTTCTGTTTA CCAGGAAGCC ATGAATCAAC CAGGCCACCT TAGACTCTTT
    TGTTGGCCTT AACCGTTCAT TTCATCTGTA CCAAACCTAT CAGCCTCCGT CAAGACAAAT GGTCCTTCGG TACTTAGTTG GTCCGGTGGA ATCTGAGAAA
```

FIG. 48C

```
                                                                                                                                        hgaI
                                                                                                                                       hinlI/acyI
                                                                                                                                       ahaII/bsaHI
                                                                                                                                    scrFI
        nlaIII                                                                                                                      mvaI      mnlI
     sau3AI                                                                                                                         ecoRII
     mboI/ndeII(dam-)                                                                                                               dsaV
     dpnI(dam+)                           maeII                                                                                     bstNI     ecoNI
     dpnII(dam-)                         aflIII                                                                                     apyI(dcm+)        mnlI
 maeIII alwI(dam-)  apoI           maeIII                                                                  mnlI                    bsaJI     bslI ddeI
901 GTGACAAGGA TCATGCAGGA ATTTGAAAGT GACACGTTTT TCCCAGAAAT TGATTTGGGG AAATATAAAC CTCTCCCAGA ATACCCAGGC GTCCTCTCTG
    CACTGTTCCT AGTACGTCCT TAAACTTTCA CTGTGCAAAA AGGGTCTTTA ACTAAACCCC TTTATATTTG GAGAGGGTCT TATGGGTCCG CAGGAGAGAC

      scrFI
      mvaI
      ecoRII
      dsaV
      bstNI
       apyI(dcm+)
    sau96I
    avaII                                                                         sfaNI
    asuI   mnlI       sfaNI                accI      mboII                       mboII                           mnlI     aluI
1001 AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTTGAAGT CTACGAGAAG AAAGACTAAC AGGAAGATGC TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT
     TCCAGGTCCT CCTTTTTCCG TAGTTCATAT TCAAACTTCA GATGCTCTTC TTTCTGATTG TCCTTCTACG AAAGTTCAAG AGACGAGGGG AGGATTTCGA
                                                                 ^END DHFR

                                                   styI
                                                   bsaJI
                                             sau3AI
                nlaIII                       mboI/ndeII(dam-)
               styI                          dpnI(dam+)                 aluI
               ncoI                          dpnII(dam-)               fnu4HI      tru9I
    ppu10I     dsaI bsmFI                    alwI(dam-)                bsoFI       mseI
    nsiI/avaIII bsaJI          cac8I        bstYI/xhoII                bbvI       aseI/asnI/vspI
1101 ATGCATTTTT ATAAGACCAT GGGACTTTTG CTGGCTTTAG ATCCCCTTGG CTTCGTTAGA ACGCAGCTAC AATTAATACA TAACCTTATG TATCATACAC
     TACGTAAAAA TATTCTGGTA CCCTGAAAAC GACCGAAATC TAGGGGAACC GAAGCAATCT TGCGTCGATG TTAATTATGT ATTGGAATAC ATAGTATGTG

                                                                          sau96I
                                                                          avaII
                                                                          asuI
                                                                        scrFI
                                                                        mvaI
                                                                        ecoRII
```

FIG. 48D

```
                                                                        dsaV
                                                                        bstNI                             ecoRI
           maeIII                                                       apyI(dcm+)                        taqI    apoI
           hphI    scfI       fokI                              bslI    bsaJI            mnlI            claI/bsp106
                                                                                         bsaJI           bspDI(dam-)
1201 ATACGATTTA GGTGACACTA TAGATAACAT CCACTTTGCC TTTCTCTCCA CAGGTGTCCA CTCCCAGGTC CAACTGCACC TCGGTTCTAT CGATTGAATT
     TATGCTAAAT CCACTGTGAT ATCTATTGTA GGTGAAACGG AAAGAGAGGT GTCCACAGGT GAGGGTCCAG GTTGACGTGG AGCCAAGATA GCTAACTTAA
                                                                   seq from pRK6G425VH: Cla-AvrII^

      nlaIII
     styI
     pflMI                                                                                            scrFI
     ncoI                                                                                             mvaI   fnu4HI
     dsaI                                                                                             ecoRII
     bslI fokI                           rmaI                                          rmaI           dsaV
     bsaJI         nlaIII   fokI         maeI             rsaI                         maeI           bstNI  bsoFI
                                         bfaI     bpmI/gsuI(dcm-)                      bfaI           apyI(dcm+)
                                                  bsrI       csp6I               aluI            haeI        bbvI
                                                                                            aciI haeIII/palI
1301 CCACCATGGG ATGGTCATGT ATCATCCTTT TTCTAGTAGC AACTGCAACT GGAGTACATT CAGAAGTTCA GCTAGTGCAG TCTGGCGGTG GCCTGGTGCA
     GGTGGTACCC TACCAGTACA TAGTAGGAAA AAGATCATCG TTGACGTTGA CCTCATGTAA GTCTTCAAGT CGATCACGTC AGACCGCCAC CGGACCACGT
   1                                                                 E  V  Q  L  V  Q  S  G  G  G  L  V  Q

                                                                                            scrFI
                                                                                            nciI
                                                                                            mspI
                                                                                            hpaII
                                                                                            dsaV
                                                                                            cauII
                                                                                           xmaI/pspAI
                                                                                           smaI
                                                                                           scrFI
                                                                                           nciI
                                                                                           dsaV          scrFI
        hgiJII                                                                             cauII         mvaI
        bsp1286                                                                            bslI          ecoRII
        bmyI                                                                               bsaJI         dsaV
     scrFI                                                                             avaI       bstNI
     mvaI banII                                         pleI                               bsaJI         bslI
     ecoRII                   aluI                hinfI                    sau96I     sau96I           apyI(dcm+)
     dsaV                    alwNI(dcm-)         taqI                      avaII      nlaIV           haeIII/palI
     bstNI                       fnu4HI              xhoI                        asuI     haeIII/palI    sau96I
     bsaJI                       bsoFI               paeR7I                     nlaIV     asuI bslI   eco0109I/draII
     apyI(dcm+)                  bbvI                avaI   maeIII          bsrI        eco0109I/draII asuI
1401 GCCAGGGGGC TCACTCCGTT TGTCCTGTGC AGCTTCTGGC TACTCCTTCT CGAGTCACTA TATGCACTGG GTCCGTCAGG CCCCGGGTAA GGGCCTGGAA
     CGGTCCCCCG AGTGAGGCAA ACAGGACACG TCGAAGACCG ATGAGGAAGA GCTCAGTGAT ATACGTGACC CAGGCAGTCC GGGGCCCATT CCCGGACCTT
  14  P  G  G  S  L  R  L  S  C  A  A  S  G  Y  S  F  S  S  H  Y  M  H  W  V  R  Q  A  P  G  K  G  L  E
```

FIG. 48E

```
                bslI
              sau3AI
              mboI/ndeII[dam-]                                                    thaI
              dpnI[dam+]                   maeII                                  fnuDII/mvnI
              dpnII[dam-]                  snaBI              haeIII/palI         bstUI
              alwI[dam-]   hphI            bsaAI              sau96I              bsh1236I
                                                              asuI               nruI
1501 TGGGTTGGAT ATATTGATCC TTCCAATGGT GAAACTACGT ATAATCAAAA GTTCAAGGGC CGTTTCACTT TATCTCGCGA CAACTCCAAA AACACAGCAT
     ACCCAACCTA TATAACTAGG AAGGTTACCA CTTTGATGCA TATTAGTTTT CAAGTTCCCG GCAAAGTGAA ATAGAGCGCT GTTGAGGTTT TTGTGTCGTA
  47 W  V  G  Y   I  D  P   S  N  G   E  T  T  Y   N  Q  K   F  K  G   R  F  T  L   S  R  D   N  S  K   N  T  A  Y

      scfI                                                                                  hinlI/acyI
      pstI                                                                                  ahaII/bsaHI
      bsgI              cac8I   mnlI                                             bsrI       aatII
     bspHI          cac8I    ddeI drdI                         mnlI             maeIII     taqI
                                                                              hphI      mboII  maeII
1601 ACCTGCAGAT GAACAGCCTG CGTGCTGAGG ACACTGCCGT CTATTACTGT GCAAGAGGGG ATTATCGCTA CAATGGTGAC TGGTTCTTCG ACGTCTGGGG
     TGGACGTCTA CTTGTCGGAC GCACGACTCC TGTGACGGCA GATAATGACA CGTTCTCCCC TAATAGCGAT GTTACCACTG ACCAAGAAGC TGCAGACCCC
  81  L  Q  M   N  S  L   R  A  E  D   T  A  V   Y  Y  C   A  R  G  D   Y  R  Y   N  G  D   W  F  F  D   V  W  G

                                                 sau96I                                                           scrFI
                                               sau96I                                                             mvaI
                                               nlaIV                                                              ecoRII
                                               hgiJII                 nlaIV                                       dsaV
                       esp3I                   bsp1286                hgiCI                                       bstNI
             scrFI                           bsp120I               banI                                        bsaJI
             mvaI      bsmBI                   bmyI                 scrFI                                     sau96I
             ecoRII                          banII             mvaI                                           haeIII/palI
             dsaV                            asuI         mboII ecoRII                                  asuI
             bstNI hphI        mnlI                 apaI      bpuAI     dsaV         hgiAI/aspHI            fnu4HI
             apyI[dcm+]        bsaJI             styI haeIII/palI       bstNI    bseRI  bsp1286                 bsoFI
            bsaJI maeIII     bseRI    mnlI   bsaJI asuI       bbsI      apyI[dcm+]  mnlI  bsiHKAI      bsp1286 aciI bsaJI
        nlaIV    bstEII bsmAI     haeIII/palI eco0109I/draII        bsaJI      mnlI    bmyI   mnlI    bmyI  nspBII apyI[dcm+]
1701 TCAAGGAACC CTGGTCACCG TCTCCTCGGC CTCCACCAAG GGCCCATCGG TCTTCCCCCT GGCACCCTCC TCCAAGAGCA CCTCTGGGGG CACAGCGGCC
     AGTTCCTTGG GACCAGTGGC AGAGGAGCCG GAGGTGGTTC CCGGGTAGCC AGAAGGGGGA CCGTGGGAGG AGGTTCTCGT GGAGACCCCC GTGTCGCCGG
 114  Q  G  T   L  V  T  V   S  S  A   S  T  K   G  P  S  V   F  P  L   A  P  S   S  K  S  T   G  G   T  A  A
```

FIG. 48F

```
               scrFI
               mvaI                                                             hinPI
               ecoRII                                                 nlaIV                   hgiAI/aspHI
               ecoNI                                                          narI               bsp1286
               dsaV                               maeIII              kasI                    bsiHKAI
               bstNI                                 hphI                     hinlI/acyI         bmyI       mspI
               bslI                                mspI                       hgiCI           cac8I        hpaII
               apyI[dcm+]                          hpaII                      haeII          fnu4HI       scrFI
           fnu4HI                                 cfr10I/bsrFI                banI           bsoFI        nciI
           bsoFI                                  bsaWI                       ahaII/bsaHI    aciI apaLI/snoI dsaV
           bbvI                        bslI       ageI  tth111I/aspI     ddeI hhaI/cfoI  nspBII alw44I/snoI cauII    scfI
1801 CTGGGCTGCC TGGTCAAGGA CTACTTCCCC GAACCGGTGA CGGTGTCGTG GAACTCAGGC GCCCTGACCA GCGGCGTGCA CACCTTCCCG GCTGTCCTAC
     GACCCGACGG ACCAGTTCCT GATGAAGGGG CTTGGCCACT GCCACAGCAC CTTGAGTCCG CGGGACTGGT CGCCGCACGT GTGGAAGGGC CGACAGGATG
147  L  G  C  L   V  K  D   Y  F  P   E  P  V  T   V  S  W   N  S  G   A  L  T  S   G  V  H   T  F  P   A  V  L  Q

                                                                fnu4HI
                                                                bsoFI     nlaIV
        ddeI pleI                 fnu4HI                      rmaI        hgiCI
       mnlI  hinfI                bsoFI              bsp1286 maeI         banI                       tfiI
       eco81I              mnlI   bbvI       maeIII           bfaI  aluI  bsp1286                    hinfI
       bsu36I/mstII/sauI   ddeI              hphI    bmyI mnlI   bbvI     bmyI                 maeII
1901 AGTCCTCAGG ACTCTACTCC CTCAGCAGCG TGGTGACTGT GCCCTCTAGC AGCTTGGGCA CCCAGACCTA CATCTGCAAC GTGAATCACA AGCCCAGCAA
     TCAGGAGTCC TGAGATGAGG GAGTCGTCGC ACCACTGACA CGGGAGATCG TCGAACCCGT GGGTCTGGAT GTAGACGTTG CACTTAGTGT TCGGGTCGTT
181   S  S  G   L  Y  S   L  S  S  V   V  T  V   P  S  S   S  L  G  T   Q  T  Y   I  C  N   V  N  H  K   P  S  N

                                                                                                ahdI/eam1105I
                                                                                               sau96I
                                                                                               avaII
                                                                                         scrFI
                                                                                         mvaI    asuI
                                                                                         ecoRII
                                 hgiJII                                                  dsaV
       styI                      bsp1286                         nlaIII                  bstNI  nlaIV        mboII
       bsaJI                     bmyI                           nspI          bsp1286    bsaJI  bsmFI        bpuAI
     mslI                        banII       maeIII             nspHI         bmyI alwNI[dcm-]  apyI[dcm+]   bbsI mnlI
2001 CACCAAGGTG GACAAGAAAG TTGAGCCCAA ATCTTGTGAC AAAACTCACA CATGCCCACC GTGCCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC
     GTGGTTCCAC CTGTTCTTTC AACTCGGGTT TAGAACACTG TTTTGAGTGT GTACGGGTGG CACGGGTCGT GGACTTGAGG ACCCCCCTGG CAGTCAGAAG
214   T  K  V   D  K  K  V   E  P  K   S  C  D   K  T  H  T   C  P  P   C  P  A   P  E  L  L   G  G  P   S  V  F
```

FIG. 48G

```
                                                 sau96I
                                                 nlaIV
                                              mspI
                                              hpaII
                                             scrFI
                                             nciI
                                             dsaV
                                        sau3AI   avaII               nlaIII
                                        mboI/ndeII[dam-]            nspI
                                    nlaIII   cauII      mnlI        nspHI                            drdI   mnlI
                                   rcaI dpnI[dam+]     ddeI        mslI                          mboII  ddeI
      mboII           styI        mnlI  dpnII[dam-]   eco81I maeIII                              bpuAI eco81I
     earI/ksp632I     bsaJI   mslI bspHI[dam-]   asuI bsu36I/mstII/sauI           maeII          bbsI  bsu36I/mstII/sauI
2101 CTCTTCCCCC CAAAACCCAA GGACACCCTC ATGATCTCCC GGACCCCTGA GGTCACATGC GTGGTGGTGG ACGTGAGCCA CGAAGACCCT GAGGTCAAGT
     GAGAAGGGGG GTTTTGGGTT CCTGTGGGAG TACTAGAGGG CCTGGGGACT CCAGTGTACG CACCACCACC TGCACTCGGT GCTTCTGGGA CTCCAGTTCA
247  L  F  P  P   K  P  K   D  T  L   M  I  S  R   T  P  E   V  T  C   V  V  V  D   V  S  H   E  D  P   E  V  K  F

                                                          aciI
                                                         thaI
                                                         fnuDII/mvnI
                                                         bstUI
                                                         bsh1236I
                                                        sacII/sstII
                                                        nspBII
                                                        kspI
                                                        dsaI
              maeII                                     bsaJI                        rsaI
            rsaI                                        aciI                         csp6I
            csp6I                                      fnu4HI  mnlI     rsaI       maeII                   hphI
        bsrI bsaAI           mnlI                      bsoFI   bseRI    csp6I      bsaAI            hgaI mnlI
2201 TCAACTGGTA CGTGGACGGC GTGGAGGTGC ATAATGCCAA GACAAAGCCG CGGGAGGAGC AGTACAACAG CACGTACCGT GTGGTCAGCG TCCTCACCGT
     AGTTGACCAT GCACCTGCCG CACCTCCACG TATTACGGTT CTGTTTCGGC GCCCTCCTCG TCATGTTGTC GTGCATGGCA CACCAGTCGC AGGAGTGGCA
281   N  W  Y   V  D  G   V  E  V  H   N  A  K   T  K  P   R  E  E  Q   Y  N  S   T  Y  R   V  V  S  V   L  T  V

           scrFI
           mvaI
           ecoRII
           dsaV                                                                                       fnu4HI
     ecoNI bstNI bsrI                    rsaI          bsmAI                                                 bsoFI
     bslI  apyI[dcm+]                    csp6I        bsaI            mnlI             taqI                  bbvI
2301 CCTGCACCAG GACTGGCTGA ATGGCAAGGA GTACAAGTGC AAGGTCTCCA ACAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCATCTCCAA AGCCAAAGGG
     GGACGTGGTC CTGACCGACT TACCGTTCCT CATGTTCACG TTCCAGAGGT TGTTTCGGGA GGGTCGGGGG TAGCTCTTTT GGTAGAGGTT TCGGTTTCCC
314   L  H  Q   D  W  L  N   G  K  E   Y  K  C   K  V  S  N   K  A  L   P  A  P   I  E  K  T   I  S  K   A  K  G
```

FIG. 48H

```
                                         scrFI
                                         nciI
                                         mspI
                                         hpaII
                                         dsaV
                                         cauII
                                        xmaI/pspAI
                                        smaI
                                        scrFI
                                        nciI
                                        dsaV
                                        cauII               scrFI          scrFI
                                                            mvaI                mvaI
                                                            ecoRII         ecoRII
                                                            dsaV           dsaV
                        rsaI          fokI                  bstNI                bstNI
                        csp6I       bslI  bsaJI mboII       apyI(dcm+)           apyI(dcm+)
      avaI            bsp1407I/bsrGI bslI avaI  earI/ksp632I sexAI          bspMI
2401 CAGCCCCGAG AACCACAGGT GTACACCCTG CCCCCATCCC GGGAAGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA
     GTCGGGGCTC TTGGTGTCCA CATGTGGGAC GGGGGTAGGG CCCTTCTCTA CTGGTTCTTG GTCCAGTCGG ACTGGACGGA CCAGTTTCCG AAGATAGGGT
347  Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N  Q  V  S  L  T  C  L  V  K  G  F  Y  P  S

                                      mspI
                                      hpaII
          dsaI                       fnu4HI
          bslI                       bsoFI                                    pleI                     mnlI
      mslI bsaJI           bsrDI     bbvI                           mnlI       hinfI      nlaIV  mboII   scfI cac8I
2501 GCGACATCGC CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTACAG
     CGCTGTAGCG GCACCTCACC CTCTCGTTAC CCGTCGGCCT CTTGTTGATG TTCTGGTGCG GAGGGCACGA CCTGAGGCTG CCGAGGAAGA AGGAGATGTC
381   D  I  A  V  E  W  E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  V  L  D  S  D  G  S  F  F  L  Y  S

                                            mboII
                                           bpuAI
          dsaI                fnu4HI     maeII                 nlaIII
       hphI                   bsoFI    xmnI bbsI             ppu10I                                  sapI
     aluI  bsaJI          bspMI bbvI   asp700        nlaIII  nsiI/avaIII                           mboII mnlI
                                                            sfaNI    mnlI                        earI/ksp632I
2601 CAAGCTCACC GTGGACAAGA GCAGGTGGCA GCAGGGGAAC GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC
     GTTCGAGTGG CACCTGTTCT CGTCCACCGT CGTCCCCTTG CAGAAGAGTA CGAGGCACTA CGTACTCCGA GACGTGTTGG TGATGTGCGT CTTCTCGGAG
414   K  L  T  V  D  K  S  R  W  Q  Q  G  N  V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L
```

FIG. 48I

```
                                                                                                 sau96I
                                                                                        aciI     haeIII/palI
             scrFI                                     taqI                            fnu4HI    asuI
             nciI                                  pleI                                bsoFI nlaIII
             mspI                               rmaI salI    scfI                       sfiI  styI
             hpaII                              maeI hincII/hindII                     eaeI   ncoI                       aluI
             dsaV                          sau96I  hinfI    pstI                       cfrI   dsaI                     fnu4HI
         bsmAI                             haeIII/palI      bsgI      aluI haeIII/palI                                 bsoFI
      bslI   cauII                         asuI bfaI accI bspMI     hindIII bglI bsaJI                                 bbvI          maeIII
2701 TCCCTGTCTC CGGGTAAATG AGTGCGACGG CCCTAGAGTC GACCTGCAGA AGCTTGGCCG CCATGGCCCA ACTTGTTTAT TGCAGCTTAT AATGGTTACA
     AGGGACAGAG GCCCATTTAC TCACGCTGCC GGGATCTCAG CTGGACGTCT TCGAACCGGC GGTACCGGGT TGAACAAATA ACGTCGAATA TTACCAATGT
 447 S  L  S  P   G  K  O

                                                                   rmaI
                                                                   maeI
              sfaNI  apoI                                    bsmI  bfaI                                                  nlaIII alwI(dam-)
2801 AATAAAGCAA TAGCATCACA AATTTCACAA ATAAAGCATT TTTTTCACTG CATTCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG
     TTATTTCGTT ATCGTAGTGT TTAAAGTGTT TATTTCGTAA AAAAAGTGAC GTAAGATCAA CACCAAACAG GTTTGAGTAG TTACATAGAA TAGTACAGAC

        sau3AI
        mboI/ndeII(dam-)
        dpnI(dam+)
        dpnII(dam-)
       pvuI/bspCI
       mcrI
       bsiEI
      taqI(dam-)  tru9I                                                           rsaI
     claI/bsp106(dam-)          fnu4HI    haeI                                     csp6I
     bspDI(dam-)  mseI          bsoFI styI                                        nlaIV
     sau3AI     xmnI            bbvI  ncoI                                        kpnI
     mboI/ndeII(dam-)         hinPI   dsaI haeIII/palI                            hgiCI
     dpnI(dam+) asp700        hhaI/cfoI nlaIII                                    banI
     dpnII(dam-)  aseI/asnI/vspI  bsaJI           mnlI        mnlI                asp718     mnlI
                                                                                  acc65I    ddeI  aciI
2901 GATCGATCGG GAATTAATTC GGCGCAGCAC CATGGCCTGA AATAACCTCT GAAAGAGGAA CTTGGTTAGG TACCTTCTGA GGCGGAAAGA ACCATCTGTG
     CTAGCTAGCC CTTAATTAAG CCGCGTCGTG GTACCGGACT TTATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCCTTTCT TGGTAGACAC
```

FIG. 48J

```
                                    scrFI                        sfaNI
                                    mvaI                             ppu10I                scrFI
                                    ecoRII                     nsiI/avaIII           mvaI
                                    dsaV                      nlaIII                 ecoRII
                                    bstNI                          sphI                    dsaV
                                    apyI[dcm+]                     nspI                    bstNI
                                   bsaJI                           nspHI                   apyI[dcm+]
                               bsmFI   nlaIV    cac8I              cac8I .                sexAI         bsmFI
3001 GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG
     CTTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC

          nlaIV                           sfaNI
        scrFI                      ppu10I
        mvaI                            nsiI/avaIII
        ecoRII                    nlaIII
        dsaV                     sphI
        bstNI                          nspI
        apyI[dcm+]                     nspHI                          aciI              aciI
       bsaJI        cac8I              cac8I                        bsmFI    aciI  fokI            aciI
3101 TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC
     AGGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG TAGAGTTAAT CAGTCGTTGG TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG

                                                                      fnu4HI
                                                                      bsoFI
                                                                      bglI
                              nlaIII                             sfiI
                             styI                                haeIII/palI
                             ncoI                              mnlI  mnlI     ddeI
                        balI dsaI                         haeIII/palI bsaJI mnlI  aluI          mnlI
        bsrI aciI       aciI bsaJI                    mnlI bsaJI aciI   haeIII/palI    bseRI
3201 CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG
     GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC CGACTGATTA AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC
```

FIG. 48K

```
                                                     scrFI
                                                     nciI
                                                     mspI
           rmaI                                      hpaII
           maeI                                      dsaV
          styI                                      haeIII/palI
          bsaJI                  aluI              mcrI                       tfiI
          blnI                  rmaI               eagI/xmaIII/eclXI          hinfI
          avrII[dam-]           maeI               eaeI                     aciI
        HaeIII/palI             bfaI               cfrI                     thaI
       stuI                    nheI                bsiEI                    fnuDII/mvnI
       haeI                    cac8I              mspI cauII                bstUI            pleI
 mnlI  mnlI bfaI              aluI                hpaII                     bsh1236I         hinfI
3301 AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTA GCTTATCCGG CCGGGAACGG TGCATTGGAA CGCGGATTCC CCGTGCCAAG AGTCAGGTAA
     TCCTCCGAAA AAACCTCCGG ATCCGAAAAC GTTTTTCGAT CGAATAGGCC GGCCCTTGCC ACGTAACCTT GCGCCTAAGG GGCACGGTTC TCAGTCCATT
               ^seq from pSVI6B5-6G4VL: AvrII - HindIII frag                     U1 matched splice donor^

                                                                                        sau3AI
                                                                                        mboI/ndeII[dam-]
                                                                                        dpnI[dam+]
                                                                                        dpnII[dam-]
                                                                                        alwI[dam-]
                                                                                      taqI[dam-]
                                                                                     claI/bsp106[dam-]
                                                   fnu4HI                            bspDI[dam-]
                                                   bsoFI                            sau3AI
                                                   aciI                             mboI/ndeII[dam-]
                        bstXI                     thaI                              dpnI[dam+]
   aciI          scfI  sau96I     styI           fnuDII/mvnI tru9I                  dpnII[dam-]
 rsaI        pleI      haeIII/palI               bstUI       mseI                  alwI[dam-]           fokI
 csp6I  scfI hinfI     asuI       bsaJI          bsh1236I    aseI/asnI/vspI
3401 GTACCGCCTA TAGAGTCTAT AGGCCCACCC CCTTGGCTTC GTTAGAACGC GGCTACAATT AATACATAAC CTTTTGGATC GATCCTACTG ACACTGACAT
     CATGGCGGAT ATCTCAGATA TCCGGGTGGG GGAACCGAAG CAATCTTGCG CCGATGTTAA TTATGTATTG GAAAACCTAG CTAGGATGAC TGTGACTGTA
                                            ^sp6 promoter                          ^removed ATG
                                                                                          ^U2 match
                                                                                    lariat consensus^
                                                                          IgG vH natural lariat restored^
```

FIG. 48L

```
                                               sau96I
                                               avaII
                                               asuI                                rmaI
                                             scrFI                             maeI                        nlaIII
                                             mvaI                                  bfaI                       styI
                                             ecoRII                 thaI   nheI          claI/bsp106     pflMI
                                             dsaV                   fnuDII/mvnI         sfaNI            ncoI
                                             bstNI                      bstUI  cac8I      fnu4HI      ecoRI      dsaI
                                             apyI[dcm+]          mnlI   bsh1236I  aluI    bsoFI  taqI  apoI      bslI fokI
                                        bslI bsaJI               bsaJI  nruI   aluI      bbvI bspDI[dam-]        bsaJI
3501 CCACTTTTTC TTTTTCTCCA CAGGTGTCCA CTCCCAGGTC CAACTGCACC TCGGTTCGCG AAGCTAGCTT GGGCTGCATC GATTGAATTC CACCATGGGA
     GGTGAAAAAG AAAAAGAGGT GTCCACAGGT GAGGGTCCAG GTTGACGTGG AGCCAAGCGC TTCGATCGAA CCCGACGTAG CTAACTTAAG GTGGTACCCT
                                                     ^cloning linker

                                                                                  aluI
                                                                                 sstI
                                                                                 sacI
                                                                                 hgiJII
                                                                                 hgiAI/aspHI
                                                                                 ecl136II
                                                                                 bsp1286
                                                                                 bsiHKAI
                                 rmaI                  rsaI                   bsmFI  bmyI
                                 maeI          bpmI/gsuI[dcm-]             bsrI  avaI           mnlI
        nlaIII    fokI           bfaI          bsrI   csp6I    ecoRV     tth111I/aspI banII  aciI
3601 TGGTCATGTA TCATCCTTTT TCTAGTAGCA ACTGCAACTG GAGTACATTC AGATATCCAG ATGACCCAGT CCCCGAGCTC CCTGTCCGCC TCTGTGGGCG
     ACCAGTACAT AGTAGGAAAA AGATCATCGT TGACGTTGAC CTCATGTAAG TCTATAGGTC TACTGGGTCA GGGGCTCGAG GGACAGGCGG AGACACCCGC
1                                                          D  I  Q   M  T  Q  S  P  S  S   L  S  A   S  V  G  D

                              scfI                                                            scrFI
                              pstI                                                            mvaI
                              bsgI                 rsaI                                       ecoRII
            hphI            sse8387I           ddeI                   maeII                   dsaV
           maeIII          bspMI             aluI  csp6I              snaBI                   bstNI     aluI
          bstEII   hphI   bspMI           hindIII       nlaIII        bsaAI      bsrI         apyI[dcm+]
3701 ATAGGGTCAC CATCACCTGC AGGTCAAGTC AAAGCTTAGT ACATGGTATA GGTGCTACGT ATTTACACTG GTATCAACAG AAACCAGGAA AAGCTCCGAA
     TATCCCAGTG GTAGTGGACG TCCAGTTCAG TTTCGAATCA TGTACCATAT CCACGATGCA TAAATGTGAC CATAGTTGTC TTTGGTCCTT TTCGAGGCTT
18    R  V  T   I  T  C   R  S  S  Q   S  L  V   H  G  I   G  A  T  Y   L  H  W   Y  Q  Q   K  P  G  K   A  P  K
```

FIG. 48M

```
                                                                         mspI
                                                                         hpaII
                                                                         bslI
                                                                        bsaWI
                                                                      sau3AI
                                                                      mboI/ndeII(dam-)
                                                                      dpnI(dam+)
                                                                      dpnII(dam-)
                                                                      alwI(dam-)                                     fnu4HI
                         tfiI                                        nlaIV                                          bsoFI
                         hinfI         bsmFI                          bstYI/xhoII                                   bbvI
                       taqI        bpmI/gsuI(dcm-)                    bamHI                                        scfI
                      claI/bsp106  pleI                               bamHI                                        pstI
                      bspDI(dam-)  hinfI                              alwI(dam-)   bsmFI                           bsgI
3801 ACTACTGATT TACAAAGTAT CCAATCGATT CTCTGGAGTC CCTTCTCGCT TCTCTGGATC CGGTTCTGGG ACGGATTTCA CTCTGACCAT CAGCAGTCTG
     TGATGACTAA ATGTTTCATA GGTTAGCTAA GAGACCTCAG GGAAGAGCGA AGAGACCTAG GCCAAGACCC TGCCTAAAGT GAGACTGGTA GTCGTCAGAC
  51 L  L  I   Y  K  V  S   N  R  F   S  G  V   P  S  R  F   S  G  S   G  S  G   T  D  F  T   L  T  I   S  S  L

                                                                              rsaI
                                                                              csp6I
                                                                              nlaIV
                                                                              kpnI styI
                                                  bsrBI                       hgiCI              sau3AI
        mboII                          rsaI       aciI                        banI bsaJI         mboI/ndeII(dam-) fnu4HI
        bpuAI                          csp6I    bsmFI                         asp718             dpnI(dam+)        bsoFI
        bbsI                           scaI   nlaIII        maeII             acc65I             dpnII(dam-)       bbvI
3901 CAGCCAGAAG ACTTCGCAAC TTATTACTGT TCACAGAGTA CTCATGTCCC GCTCACGTTT GGACAGGGTA CCAAGGTGGA GATCAAACGA ACTGTGGCTG
     GTCGGTCTTC TGAAGCGTTG AATAATGACA AGTGTCTCAT GAGTACAGGG CGAGTGCAAA CCTGTCCCAT GGTTCCACCT CTAGTTTGCT TGACACCGAC
  84 Q  P  E  D   F  A  T   Y  Y  C   S  Q  S  T   H  V  P   L  T  F   G  Q  G  T   K  V  E   I  K  R   T  V  A  A

        mboII                                                                                                 haeIII/palI
       bpuAI                                    xmnI                         xmnI                             haeI
       bbsI     mboII aciI                      asp700                cac8I  asp700                          mnlI
4001 CACCATCTGT CTTCATCTTC CCGCCATCTG ATGAGCAGTT GAAATCTGGA ACTGCTTCTG TTGTGTGCCT GCTGAATAAC TTCTATCCCA GAGAGGCCAA
     GTGGTAGACA GAAGTAGAAG GGCGGTAGAC TACTCGTCAA CTTTAGACCT TGACGAAGAC AACACACGGA CGACTTATTG AAGATAGGGT CTCTCCGGTT
 118  P  S  V   F  I  F   P  P  S  D   E  Q  L   K  S  G   T  A  S  V   V  C  L   L  N  N   F  Y  P  R   E  A  K
```

FIG. 48N

```
                                                     scrFI
                                                     mvaI
                                                     ecoRII
                                                     dsaV
                                                     bstNI
      rsaI              mnlI                         apyI[dcm+]                                                          fnu4HI
      csp6I             bslI              maeIII bsaJI      maeIII                                          ddeI bsoFI
                                                                                                    scfI   mnlI  bbvI
4101 AGTACAGTGG AAGGTGGATA ACGCCCTCCA ATCGGGTAAC TCCCAGGAGA GTGTCACAGA GCAGGACAGC AAGGACAGCA CCTACAGCCT CAGCAGCACC
     TCATGTCACC TTCCACCTAT TGCGGGAGGT TAGCCCATTG AGGGTCCTCT CACAGTGTCT CGTCCTGTCG TTCCTGTCGT GGATGTCGGA GTCGTCGTGG
151  V  Q  W   K  V  D  N   A  L  Q   S  G  N   S  Q  E  S   V  T  E   Q  D  S   K  D  S  T   Y  S  L   S  S  T

                                                                                     sstI
                                                                                     sacI
                                                                                     hgiJII
                                                                                     hgiAI/aspHI
                                                                                     ecl136II
                                                                                     bsp1286
                                                                                     bsiHKAI
                                                                                     bmyI
                                                                                   ddeI cac8I
                                                                                haeIII/palI
          ddeI                                                                 sau96I aluI
         celII/espI                                                            asuI  banII
         blpI/bpu1102I                                           hphI         eco0109I/draII
      hgaI                                   acoI     cac8I     maeIII       alwNI[dcm-]          maeIII    aluI
4201 CTGACGCTGA GCAAAGCAGA CTACGAGAAA CACAAAGTCT ACGCCTGCGA AGTCACCCAT CAGGGCCTGA GCTCGCCCGT CACAAAGAGC TTCAACAGGG
     GACTGCGACT CGTTTCGTCT GATGCTCTTT GTGTTTCAGA TGCGGACGCT TCAGTGGGTA GTCCCGGACT CGAGCGGGCA GTGTTTCTCG AAGTTGTCCC
184  L  T  L  S   K  A  D   Y  E  K   H  K  V  Y   A  C  E   V  T  H   Q  G  L  S   S  P  V   T  K  S   F  N  R  G

                        sau96I
                  aciI  haeIII/palI
                 fnu4HI  asuI
                 bsoFI nlaIII
                 sfiI  styI
             aluI haeIII/palI                      aluI
           hindIII bglI ncoI                      fnu4HI
          tru9I   eaeI  dsaI                      bsoFI
          maeI    cfrI  bsaJI                     bbvI      maeIII                   sfaNI  apoI
4301 GAGAGTGTTA AGCTTGGCCG CCATGGCCCA ACTTGTTTAT TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA AATTTCACAA ATAAAGCATT
     CTCTCACAAT TCGAACCGGC GGTACCGGGT TGAACAAATA ACGTCGAATA TTACCAATGT TTATTTCGTT ATCGTAGTGT TTAAAGTGTT TATTTCGTAA
218   E  C  O
```

FIG. 48O

```
                                                                         sau3AI
                                                                         mboI/ndeII(dam-)
                                                                         dpnI(dam+)
                                                                         dpnII(dam-)
                                                                        pvuI/bspCI
                                                                        mcrI
                                                                        bsiEI
                                                                       taqI(dam-)
                                                                      claI/bsp106(dam-)
                                                                      bspDI(dam-)  tru9I      fnu4HI    haeI
                                                                     sau3AI        mseI       bsoFI styI
                                                                     mboI/ndeII(dam-)         bbvI  ncoI
            rmaI                                                     dpnI(dam+) xmnI         hinPI  dsaI haeIII/palI
            maeI                                                     dpnII(dam-)  aseI/asnI/vspI    bsaJI
        bsmI bfaI                                         nlaIII alwI(dam-)  asp700          hhaI/cfoI nlaIII
4401 TTTTTCACTG CATTCTAGTT GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCTG GATCGATCGG GAATTAATTC GGCGCAGCAC CATGGCCTGA
     AAAAAGTGAC GTAAGATCAA CACCAAACAG GTTTGAGTAG TTACATAGAA TAGTACAGAC CTAGCTAGCC CTTAATTAAG CCGCGTCGTG GTACCGGACT
                                                                                ^sv40 origin

                                 rsaI                                                                   scrFI
                                 csp6I                                                                  mvaI
                                nlaIV                                                                   ecoRII
                                kpnI                                                                    dsaV
                                hgiCI                                                                   bstNI
                                banI                         aluI                                       apyI(dcm+)
                                asp718     mnlI             pvuII                                      bsaJI
       mnlI       mnlI          acc65I   ddeI   aciI        nspBII                                  bsmFI   nlaIV
4501 AATAACCTCT GAAAGAGGAA CTTGGTTAGG TACCTTCTGA GGCGGAAAGA ACCAGCTGTG GAATGTGTGT CAGTTAGGGT GTGGAAAGTC CCCAGGCTCC
     TTATTGGAGA CTTTCTCCTT GAACCAATCC ATGGAAGACT CCGCCTTTCT TGGTCGACAC CTTACACACA GTCAATCCCA CACCTTTCAG GGGTCCGAGG

                              sfaNI                                     scrFI                           sfaNI
                             ppu10I                 scrFI        mvaI                                  ppu10I
                             nsiI/avaIII            mvaI               ecoRII                          nsiI/avaIII
                            nlaIII                  ecoRII       dsaV                            nlaIII
                           sphI                     dsaV         bstNI                               sphI
                           nspI                     bstNI               apyI(dcm+)                       nspI
                           nspHI                    apyI(dcm+)         bsaJI                             nspHI
       cac8I               cac8I                   sexAI          bsmFI    nlaIV      cac8I              cac8I
4601 CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAG GTGTGGAAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC
     GGTCGTCCGT CTTCATACGT TTCGTACGTA GAGTTAATCA GTCGTTGGTC CACACCTTTC AGGGGTCCGA GGGGTCGTCC GTCTTCATAC GTTTCGTACG
```

FIG. 48P

```
                                                                                                   nlaIII
                                                                                                  styI
                                                                                                  ncoI
                                 aciI                   aciI                                 bslI dsaI
                              bsmFI           aciI  fokI             aciI bsrI  aciI         aciI bsaJI
4701 ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT
     TAGAGTTAAT CAGTCGTTGG TATCAGGGCG GGGATTGAGG CGGGTAGGGC GGGGATTGAG GCGGGTCAAG GCGGGTAAGA GGCGGGGTAC CGACTGATTA

                                                                                           rmaI
                                                                                           maeI
                                fnu4HI                                                    styI
                                bsoFI                                                     bsaJI
                                bglI                                                      blnI
                               sfiI                                                       avrII[dam-]
                               haeIII/palI                                              haeIII/palI
                             mnlI   mnlI      ddeI                       mnlI          stuI
                        haeIII/palI bsaJI  mnlI   aluI               mnlI              haeI                      maeIII
                      mnlI bsaJI aciI    haeIII/palI                 bseRI            mnlI bfaI             aluI
4801 TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTG
     AAAAAAATAA ATACGTCTCC GGCTCCGGCG GAGCCGGAGA CTCGATAAGG TCTTCATCAC TCCTCCGAAA AAACCTCCGG ATCCGAAAAC GTTTTTCGAC
                                                                                                start pUC118^

                  fnu4HI
                 haeIII/palI       hinPI
                mcrI               hhaI/cfoI
                eagI/xmaIII/eclXI thaI
                eaeI              fnuDII/mvnI
              notI                bstUI                                                                         scrFI
            bsrBI bsoFI          hinPI               bspHI                                                      mvaI
          taqI  cfrI             hhaI/cfoI         scfI                                                         ecoRII
         xhoI fnu4HI   tru9I     cac8I     tru9I   pstI                    haeIII/palI                          dsaV
         paeR7I bsiEI  pacI     ascI      ahaIII/draI                     eaeI                                  bstNI
         avaI bsoFI    mseI tru9I bsh1236I mseI    bsgI  maeIII           cfrI                      bsrI        apyI[dcm+]
        mnlI  aciI aciI     mseI bssHII  swaI     sse8387I     aluI     bsrI               maeII maeIII        bsaJI
4901 TTACCTCGAG CGGCCGCTTA ATTAAGGCGC GCCATTTAAA TCCTGCAGGT AACAGCTTGG CACTGGCCGT CGTTTTACAA CGTCGTGACT GGGAAAACCC
     AATGGAGCTC GCCGGCGAAT TAATTCCGCG CGGTAAATTT AGGACGTCCA TTGTCGAACC GTGACCGGCA GCAAAATGTT GCAGCACTGA CCCTTTTGGG
       ^linearization linker inserted into Hpal site
```

FIG. 48Q

```
                                                                                                  sau3AI
                                                                                                  mboI/ndeII[dam-]
                                                                                        sau96I    dpnI[dam+]
                                                                    cac8I               haeIII/palI
                                                                    aluI                asuI      dpnII[dam-]
                                        fnu4HI                     pvuII              mnlI aciI  pvuI/bspCI
                         tru9I          bsoFI                      nspBII            mboII cac8I  mcrI
          maeIII         mseI           bbvI     fokI             cac8I              earI/ksp632I bsiEI
5001 TGGCGTTACC CAACTTAATC GCCTTGCAGC ACATCCCCCC TTCGCCAGCT GGCGTAATAG CGAAGAGGCC CGCACCGATC GCCCTTCCCA ACAGTTGCGT
     ACCGCAATGG GTTGAATTAG CGGAACGTCG TGTAGGGGGG AAGCGGTCGA CCGCATTATC GCTTCTCCGG GCGTGGCTAG CGGGAAGGGT TGTCAACGCA

                           hinPI
                           hhaI/cfoI
                          nlaIV
                          narI
                          kasI                                                                                    hinPI
                          hinlI/acyI                                                                              thaI
                          hgiCI                                                                                   fnuDII/mvnI
                          haeII        aciI                                                                       bstUI scfI
                          banI       sfaNI                                                                        bsh1236I
       bglI               ahaII/bsaHI                        sfaNI     aciI           aciI    maeII              rsaI hhaI/cfoI
5101 AGCCTGAATG GCGAATGGCG CCTGATGCGG TATTTTCTCC TTACGCATCT GTGCGGTATT TCACACCGCA TACGTCAAAG CAACCATAGT ACGCGCCCTG
     TCGGACTTAC CGCTTACCGC GGACTACGCC ATAAAAGAGG AATGCGTAGA CACGCCATAA AGTGTGGCGT ATGCAGTTTC GTTGGTATCA TGCGCGGGAC
                                                                                                          csp6I   bslI

                             aciI
                           fnu4HI                fnu4HI
                           bsoFI                 bsoFI
                          thaI                  hinPI                                     hinPI
                          fnuDII/mvnI           hhaI/cfoI                                 hhaI/cfoI
         hinPI            bstUI                thaI                                   rmaI
         hhaI/cfoI hinPI                       fnuDII/mvnI                        hinPI   haeII
      fnu4HI              hhaI/cfoI            bstUI                              hhaI/cfoI   bsrBI
      bsoFI          tru9I aciI                bsh1236I      aciI                 haeII maeI  aciI
      aciI           mseI bsh1236I          maeIII bbvI   maeIII          cac8I      bfaI  cac8I         mboII
5201 TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT GGTTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC
     ATCGCCGCGT AATTCGCGCC GCCCACACCA CCAATGCGCG TCGCACTGGC GATGTGAACG GTCGCGGGAT CGCGGGCGAG GAAAGCGAAA GAAGGGAAGG
```

FIG. 48R

```
                      mspI                             nlaIV
                      hpaII                          hgiJII
                     naeI                            bsp1286                              nlaIV
                     cfr10I/bsrFI                    bmyI                                 hgiCI  taqI
              maeII  cac8I             aluI          banII         nlaIV                  banI mnlI
5301 TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT CAAGCTCTAA ATCGGGGGCT CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC CCCAAAAAAC
     AAAGAGCGGT GCAAGCGGCC GAAAGGGGCA GTTCGAGATT TAGCCCCCGA GGGAAATCCC AAGGCTAAAT CACGAAATGC CGTGGAGCTG GGGTTTTTTG

                         maeII    haeIII/palI
                        draIII   sau96I                                 maeII pleI           tru9I      pleI
             hphI       bsaAI    asuI                                  drdI   hinfI  maeII   mseI       hinfI
5401 TTGATTTGGG TGATGGTTCA CGTAGTGGGC CATCGCCCTG ATAGACGGTT TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT
     AACTAAACCC ACTACCAAGT GCATCACCCG GTAGCGGGAC TATCTGCCAA AAAGCGGGAA ACTGCAACCT CAGGTGCAAG AAATTATCAC CTGAGAACAA

                                                                                           tru9I
                             bslI                                                          mseI          tru9I
         bsrI               bslI  avaI                                          haeIII/palI           aluI    mseI
5501 CCAAACTGGA ACAACACTCA ACCCTATCTC GGGCTATTCT TTTGATTTAT AAGGGATTTT GCCGATTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA
     GGTTTGACCT TGTTGTGAGT TGGGATAGAG CCCGATAAGA AAACTAAATA TTCCCTAAAA CGGCTAAAGC CGGATAACCA ATTTTTTACT CGACTAAATT

                                                            hgiAI/aspHI
                 thaI                                       bsp1286
                 fnuDII/mvnI             maeII              bsiHKAI                        aciI
            tru9I   apoI tru9I          psp1406I            bmyI   ddeI                   fnu4HI
            mseI bstUI   mseI        tru9I                  apaLI/snoI  rsaI              bsoFI     tru9I
        apoI     bsh1236I        sspI mseI                  alw44I/snoI csp6I          sfaNI        mseI        aciI
5601 CAAAAATTTA ACGCGAATTT TAACAAAATA TTAACGTTTA CAATTTTATG GTGCACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAACTCC
     GTTTTTAAAT TGCGCTTAAA ATTGTTTTAT AATTGCAAAT GTTAAAATAC CACGTGAGAG TCATGTTAGA CGAGACTACG GCGTATCAAT TCGGTTGAGG

                                                                    hinPI                    sfaNI
                                                                    hhaI/cfoI             mspI
                                   hinPI                           thaI                    hpaII
                                fnu4HI                             fnuDII/mvnI            scrFI
                 maeIII         bsoFI                              bstUI                  nciI
              maeII bsrI    nlaIII hhaI/cfoI                nspBII bsh1236I               dsaV fokI
             bsaAI tth111I/aspI bbvI             aciI       aciI hgaI      drdI           cauII   aciI
5701 GCTATCGCTA CGTGACTGGG TCATGGCTGC GCCCCGACAC CCGCCAACAC CCGCTGACGC GCCCTGACGG GCTTGTCTGC TCCCGGCATC CGCTTACAGA
     CGATAGCGAT GCACTGACCC AGTACCGACG CGGGGCTGTG GGCGGTTGTG GGCGACTGCG CGGGACTGCC CGAACAGACG AGGGCCGTAG GCGAATGTCT
```

FIG. 48S

```
                                                                         thaI
                      scrFI                                              fnuDII/mvnI
                      nciI                                               bstUI
                      mspI                                               bsh1236I
                      hpaII     nspI                                    hinPI
                      dsaV      nspHI                                   hhaI/cfoI                mnlI
                 esp3I       fnu4HI                                    thaI mnlI               haeIII/palI
                 bsmBI       bsoFI                                     fnuDII/mvnI         mboII      sau96I
           maeIII bsmAI      bbvI                                      bstUI               bpuAI      asuI bssSI
       aluI     bslI  cauII aluI nlaIII    mnlI    hphI      hphI      bsh1236I            bbsI      eco0109I/draII
5801 CAAGCTGTGA CCGTCTCCGG GAGCTGCATG TGTCAGAGGT TTTCACCGTC ATCACCGAAA CGCGCGAGGC AGTATTCTTG AAGACGAAAG GGCCTCGTGA
     GTTCGACACT GGCAGAGGCC CTCGACGTAC ACAGTCTCCA AAAGTGGCAG TAGTGGCTTT GCGCGCTCCG TCATAAGAAC TTCTGCTTTC CCGGAGCACT

                                                                                             nlaIV
                                                                                          aciI
                                                                                         thaI
                                                                                         fnuDII/mvnI
                                                       hinlI/acyI                        bstUI
                                nlaIII                 ahaII/bsaHI                       bsh1236I
                        tru9I  rcaI                    aatII                            hinPI
                        mseI   bspHI               ddeI maeII                           hhaI/cfoI
5901 TACGCCTATT TTTATAGGTT AATGTCATGA TAATAATGGT TTCTTAGACG TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA TTTGTTTATT
     ATGCGGATAA AAATATCCAA TTACAGTACT ATTATTACCA AAGAATCTGC AGTCCACCGT GAAAAGCCCC TTTACACGCG CCTTGGGGAT AAACAAATAA

                                    rcaI
                                    bspHI
                                bsrBI    bsmAI                                         mboII
                                aciI  nlaIII                             sspI          earI/ksp632I         msII
6001 TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT TCAACATTTC
     AAAGATTTAT GTAAGTTTAT ACATAGGCGA GTACTCTGTT ATTGGGACTA TTTACGAAGT TATTATAACT TTTTCCTTCT CATACTCATA AGTTGTAAAG

                                                                                                               hgiAI/aspHI
                                                                                                               bsp1286
                                                                                                    sau3AI     bsiHKAI
                                                                                                    mboI/ndeII(dam-)
                                                                                                    dpnI(dam+) bmyI
                               fnu4HI                                                               dpnII(dam-)
                               bsoFI                                                           eco57I          apaLI/snoI
                               aciI                         hphI            hphI          sfaNI  mboII(dam-)   alw44I/snoI
6101 CGTGTCGCCC TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC TCACCCAGAA ACGCTGGTGA AAGTAAAAGA TGCTGAAGAT CAGTTGGGTG
     GCACAGCGGG AATAAGGGAA AAAACGCCGT AAAACGGAAG GACAAAAACG AGTGGGTCTT TGCGACCACT TTCATTTTCT ACGACTTCTA GTCAACCCAC
```

FIG. 48T

```
                         sau3AI    nspBII     sau3AI
                         mboI/ndeII[dam-]     mboI/ndeII[dam-]            maeII
                         dpnI[dam+]           dpnI[dam+]                 psp1406I          hgiAI/aspHI
                        bstYI/xhoII           dpnII[dam-]                xmnI              bsp1286  tru9I
                    bsrI dpnII[dam-]          alwI[dam-]                 asp700            bsiHKAI  mseI
     bssSI   maeIII taqI   alwI[dam-]  aciI  bstYI/xhoII             mboII                 bmyI   ahaIII/draI
6201 CACGAGTGGG TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT TTAAAGTTCT
     GTGCTCACCC AATGTAGCTT GACCTAGAGT TGTCGCCATT CTAGGAACTC TCAAAAGCGG GGCTTCTTGC AAAAGGTTAC TACTCGTGAA AATTTCAAGA

                                   scrFI
           aciI                    nciI
          thaI                     mspI
          fnuDII/mvnI              hpaII
          bstUI                    dsaV
          bsh1236I              hinlI/acyI                aciI                            rsaI
         hinPI                  hgaI cauII          mcrI  fnu4HI                          csp6I   bsrI
         hhaI/cfoI              ahaII/bsaHI   bcgI  bsiEI bsoFI          ddeI             scaI  hphI  maeIII
6301 GCTATGTGGC GCGGTATTAT CCCGTGATGA CGCCGGGCAA GAGCAACTCG GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA CTCACCAGTC
     CGATACACCG CGCCATAATA GGGCACTACT GCGGCCCGTT CTCGTTGAGC CAGCGGCGTA TGTGATAAGA GTCTTACTGA ACCAACTCAT GAGTGGTCAG

                                                                                                     sau3AI
                                                                           haeIII/palI               mboI/ndeII[dam-]
                                                                          eaeI                       dpnI[dam+]
                                                                          cfrI                       dpnII[dam-]
                                                 fnu4HI                  fnu4HI                     pvuI/bspCI
                                                 bsoFI                   bsoFI                      mcrI
         sfaNI    fokI   nlaIII                  bbvI  mslI  nlaIII      aciI                       bsiEI
6401 ACAGAAAAGC ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC TGCCATAACC ATGAGTGATA ACACTGCGGC CAACTTACTT CTGACAACGA
     TGTCTTTTCG TAGAATGCCT ACCGTACTGT CATTCTCTTA ATACGTCACG ACGGTATTGG TACTCACTAT TGTGACGCCG GTTGAATGAA GACTGTTGCT

                                                nlaIII
                                              sau3AI maeIII                        mspI
        sau96I                                mboI/ndeII[dam-]    sau3AI    nlaIV
        avaII                                 dpnI[dam+]          mboI/ndeII[dam-] aluI
        asuI                                  dpnII[dam-]         dpnI[dam+]     hpaII
      mnlI         aluI    aciI          nlaIII alwI[dam-]        dpnII[dam-] bsaWI
6501 TCGGAGGACC GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAAA
     AGCCTCCTGG CTTCCTCGAT TGGCGAAAAA ACGTGTTGTA CCCCCTAGTA CATTGAGCGG AACTAGCAAC CCTTGGCCTC GACTTACTTC GGTATGGTTT
```

FIG. 48U

```
                                                     hinPI                                       mspI
                                                     hhaI/cfoI                                   hpaII
                                fnu4HI               mstI                                   aluI scrFI
                                bsoFI                aviII/fspI    bsrI                   rmaI   nciI        tru9I
                  mslI     cac8I  bsrDI          maeII          tru9I                     maeI   dsaV        mseI
             maeIII    sfaNI    bbvI           psp1406I         mseI                      bfaI   cauII      aseI/asnI/vspI
6601 CGACGAGCGT GACACCACGA TGCCAGCAGC AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA ACAATTAATA
     GCTGCTCGCA CTGTGGTGCT ACGGTCGTCG TTACCGTTGT TGCAACGCGT TTGATAATTG ACCGCTTGAT GAATGAGATC GAAGGGCCGT TGTTAATTAT

                                                      bglI                                       mspI
                                                     sau96I    cac8I                             hpaII
                                 sau96I              haeIII/palI                                cfr10I/bsrFI
         fokI     aciI           avaII        hinPI  asuI   mspI                            nlaIV hphI        bsmAI
      bsrI    mnlI               asuI         hhaI/cfoI     hpaII                         bpmI/gsuI(dcm-)    bsaI
6701 GACTGGATGG AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT GAGCGTGGGT
     CTGACCTACC TCCGCCTATT TCAACGTCCT GGTGAAGACG CGAGCCGGGA AGGCCGACCG ACCAAATAAC GACTATTTAG ACCTCGGCCA CTCGCACCCA

        aciI
       thaI         fnu4HI     haeIII/palI
       fnuDII/mvnI  bsoFI     sau96I                                                 pleI
       bstUI        bbvI     nlaIV                                                   hinfI
       bsh1236I bsrDI   bsrI asuI             mnlI                          ahdI/eam1105I          fokI
6801 CTCGCGGTAT CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAAATAG
     GAGCGCCATA GTAACGTCGT GACCCCGGTC TACCATTCGG GAGGGCATAG CATCAATAGA TGTGCTGCCC CTCAGTCCGT TGATACCTAC TTGCTTTATC

             ddeI
        sau3AI        nlaIV
        mboI/ndeII(dam-)                                                                      tru9I
        dpnI(dam+)    hgiCI         tru9I                                                     mseI            tru9I
        dpnII(dam-)   banI mnlI     mseI        maeIII                                       ahaIII/draI      mseI
6901 ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAAACT TCATTTTTAA
     TGTCTAGCGA CTCTATCCAC GGAGTGACTA ATTCGTAACC ATTGACAGTC TGGTTCAAAT GAGTATATAT GAAATCTAAC TAAATTTTGA AGTAAAAATT

                rmaI
                maeI      sau3AI
            sau3AI hphI   mboI/ndeII(dam-)
            mboI/ndeII(dam-)
            dpnI(dam+)    dpnI(dam+)
            dpnII(dam-)   dpnII(dam-)                                                                         sau3AI
      tru9I bstYI/xhoII   alwI(dam-)       nlaIII              maeII                                         mboI/ndeII(dam-)
      mseI  alwI(dam-)  bstYI/xhoII       rcaI              tru9I                      hgaI                  dpnI(dam+)
     ahaIII/draI bfaI mboII(dam-)         bspHI             mseI                   ddeI                      dpnII(dam-)
7001 TTTAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA
     AAATTTTCCT AGATCCACTT CTAGGAAAAA CTATTAGAGT ACTGGTTTTA GGGAATTGCA CTCAAAAGCA AGGTGACTCG CAGTCTGGGG CATCTTTTCT
```

FIG. 48V

```
                       sau3AI
             mboII(dam-)                                                                  sau3AI
           sau3AI      mboI/ndeII(dam-) thaI                                              mboI/ndeII(dam-)
           mboI/ndeII(dam-)             fnuDII/mvnI                                       dpnI(dam+)
           dpnI(dam+)  dpnI(dam+)       bstUI         cac8I                               dpnII(dam-)
           dpnII(dam-) dpnII(dam-)      bsh1236I   fnu4HI                                alwI(dam-)
          bstYI/xhoII  alwI(dam-)      hinPI       bsoFI                   aciI         mspI
          alwI(dam-) bstYI/xhoII       hhaI/cfoI   bbvI               aciI    nspBII    hpaII      aluI
7101 TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA
     AGTTTCCTAG AAGAACTCTA GGAAAAAAAG ACGCGCATTA GACGACGAAC GTTTGTTTTT TTGGTGGCGA TGGTCGCCAC CAAACAAACG GCCTAGTTCT

                                                                          rmaI
                                bsrI            hinPI                     maeI                haeIII/palI
                             maeIII    eco57I   hhaI/cfoI                 bfaI      bslI     haeI
7201 GCTACCAACT CTTTTTCCGA AGGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA CTTCAAGAAC
     CGATGGTTGA GAAAAAGGCT TCCATTGACC GAAGTCGTCT CGCGTCTATG GTTTATGACA GGAAGATCAC ATCGGCATCA ATCCGGTGGT GAAGTTCTTG

                                                            fnu4HI
                                                            bsoFI                         scrFI
                                                            bbvI                          nciI
                                                        fnu4HI                            mspI
                                                   alwNI(dcm-)                            hpaII
                                                  bsrI  bsoFI                             dsaV     pleI
      scfI    aciI         mnlI                  maeIII     bbvI    bsrI                  cauII    hinfI
7301 TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT
     AGACATCGTG GCGGATGTAT GGAGCGAGAC GATTAGGACA ATGGTCACCG ACGACGGTCA CCGCTATTCA GCACAGAATG GCCCAACCTG AGTTCTGCTA

                         aciI
                       nspBII                       hgiAI/aspHI
                      fnu4HI                        bsp1286
         mspI         bsoFI                         bsiHKAI
         hpaII        bbvI mcrI                     bmyI
        bsaWI       hinPI  bsiEI                    apaLI/snoI
      maeIII        hhaI/cfoI                       alw44I/snoI   aluI                          ddeI      scfI
7401 AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG
     TCAATGGCCT ATTCCGCGTC GCCAGCCCGA CTTGCCCCCC AAGCACGTGT GTCGGGTCGA ACCTCGCTTG CTGGATGTGG CTTGACTCTA TGGATGTCGC
```

FIG. 48W

```
                                                                                                                           scrFI
                                                                                                                           mvaI
                                                                                                                           ecoRII
                                                               mspI                                                        dsaV
          hinPI                                                hpaII  fnu4HI                               bssSI           bstNI
           hhaI/cfoI                                           bslI   bsoFI                             hinPI mnlI         bsaJI
          haeII                                    aciI        bsaWI  aciI                              hhaI/cfoI    aluI  apyI[dcm+]
7501 TGAGCATTGA GAAAGCGCCA CGCTTCCCGA AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG GGAGCTTCCA
     ACTCGTAACT CTTTCGCGGT GCGAAGGGCT TCCCTCTTTC CGCCTGTCCA TAGGCCATTC GCCGTCCCAG CCTTGTCCTC TCGCGTGCTC CCTCGAAGGT

          scrFI
          mvaI
          ecoRII
          dsaV
          bstNI                                                       taqI                                 nlaIV
          apyI[dcm+]                              mnlI drdI        hgaI              sfaNI            aciI
7601 GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA
     CCCCCTTTGC GGACCATAGA AATATCAGGA CAGCCCAAAG CGGTGGAGAC TGAACTCGCA GCTAAAAACA CTACGAGCAG TCCCCCCGCC TCGGATACCT

              haeIII/palI          haeIII/palI
            fnu4HI               scrFI
            bsoFI                mvaI bslI
            aciI                 ecoRII
           thaI bslI             dsaV                       nlaIII
           fnuDII/mvnI           bstNI                haeIII/palI  nspI
           bstUI                 apyI[dcm+]            haeI        nspHI                        tfiI
      cac8I bsh1236I        nlaIV haeI              cac8I          aflIII                       hinfI
7701 AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC TGCGTTATCC CCTGATTCTG TGGATAACCG
     TTTTGCGGTC GTTGCGCCGG AAAAATGCCA AGGACCGGAA AACGACCGGA AAACGAGTGT ACAAGAAAGG ACGCAATAGG GGACTAAGAC ACCTATTGGC

                                 fnu4HI
                                 bsoFI                  fnu4HI
                                 bbvI                   bsoFI                         sapI  hinPI
                             cac8I aciI                 bbvI pleI                     mboII hhaI/cfoI
                           bsrBI  fnu4HI       mcrI    hinPI  hinfI                   earI/ksp632I
      aciI          aluI    aciI   bsoFI       bsiEI   hhaI/cfoI               mnlI  aciI   haeII
7801 TATTACCGCC TTTGAGTGAG CTGATACCGC TCGCCGCAGC CGAACGACCG AGCGCAGCGA GTCAGTGAGC GAGGAAGCGG AAGAGCGCCC AATACGCAAA
     ATAATGGCGG AAACTCACTC GACTATGGCG AGCGGCGTCG GCTTGCTGGC TCGCGTCGCT CAGTCACTCG CTCCTTCGCC TTCTCGCGGG TTATGCGTTT
```

FIG. 48X

```
                  thaI
                  fnuDII/mvnI
                  bstUI
                  bsh1236I
                 hinPI
                 hhaI/cfoI
                thaI
                fnuDII/mvnI                 cac8I
                bstUI   haeIII/palI        aluI
                bsh1236I           tru9I  pvuII                                                 tru9I
       mnlI    bslI    eaeI  tfiI aseI/asnI/vspI                          cac8I      hinPI      mseI      maeIII
    aciI       aciI    cfrI  hinfI mseI   nspBII                  bsrI     aciI       hhaI/cfoI aseI/asnI/vspI
7901 CCGCCTCTCC CCGCGCGTTG GCCGATTCAT TAATCCAGCT GGCACGACAG GTTTCCCGAC TGGAAAGCGG GCAGTGAGCG CAACGCAATT AATGTGAGTT
     GGCGGAGAGG GGCGCGCAAC CGGCTAAGTA ATTAGGTCGA CCGTGCTGTC CAAAGGGCTG ACCTTTCGCC CGTCACTCGC GTTGCGTTAA TTACACTCAA

                        scrFI
                        mvaI
                        ecoRII
                        dsaV
                  nlaIV bstNI
                  hgiCI apyI[dcm+]        mspI                        aciI
     mnlI         banI bsaJI              hpaII                     bsrBI                          aluI
8001 ACCTCACTCA TTAGGCACCC CAGGCTTTAC ACTTTATGCT TCCGGCTCGT ATGTTGTGTG GAATTGTGAG CGGATAACAA TTTCACACAG GAAACAGCTA
     TGGAGTGAGT AATCCGTGGG GTCCGAAATG TGAAATACGA AGGCCGAGCA TACAACACAC CTTAACACTC GCCTATTGTT AAAGTGTGTC CTTTGTCGAT
```

FIG. 48Y

```
                    tru9I
                    mseI
                   aseI/asnI/vspI
                  xmnI
        nlaIII    asp700
  8101 TGACCATGAT TACGAATTAA
       ACTGGTACTA ATGCTTAATT

>length: 8120

aatII(GACGTC):          1690 5947
acc65I(GGTACC):         2969 3967 4529
accI(GTMKAC):           823 1039 2738 4237
aciI(CCGC):             217 229 238 250 260 271 317 422 454 485 574 1385 1795 1871 2248 2250 2758 2982
                        3167 3179 3188 3200 3210 3221 3267 3372 3404 3449 3686 3949 4021 4318 4542 4727
                        4739 4748 4760 4770 4781 4827 4910 4914 5070 5127 5153 5166 5203 5217 5220 5248
                        5275 5680 5699 5741 5751 5790 5979 6026 6125 6234 6311 6355 6476 6522 6713 6804
                        7166 7175 7310 7420 7541 7560 7687 7715 7806 7827 7834 7877 7901 7911 7967 8070
acyI                    see hinlI
aflII/bfrI(CTTAAG):     786
aflIII(ACRYGT):         932 7758
ageI(ACCGGT):           1833
ahaII/bsaHI(GRCGYC):    988 1690 1858 5117 5947 6329
ahaIII/draI(TTTAAA):    696 4935 6290 6982 7001
ahdI/eam1105I(GACNNNNNGTC): 2087 6865
aluI(AGCT):             5 44 332 386 390 753 1097 1165 1370 1431 1951 2603 2751 2784 3282 3336 3340
                        3562 3566 3676 3733 3792 4270 4288 4311 4344 4554 4842 4896 4954 5047 5333 5590
                        5803 5822 6516 6579 6679 7200 7457 7593 7819 7937 8096
alw44I/snoI(GTGCAC):    1876 5651 6198 7444
```

FIG. 48Z

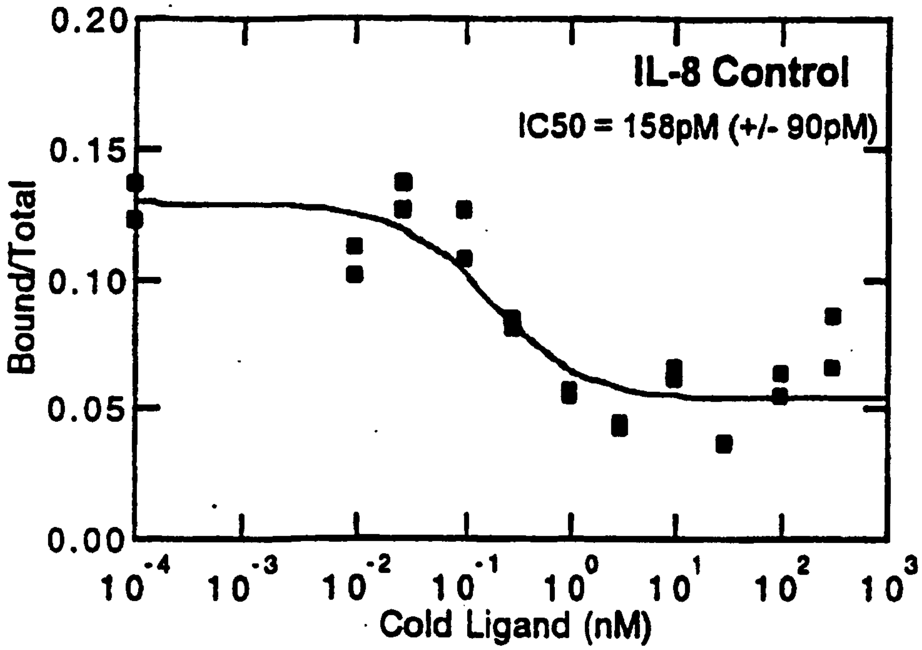


FIG. 49A

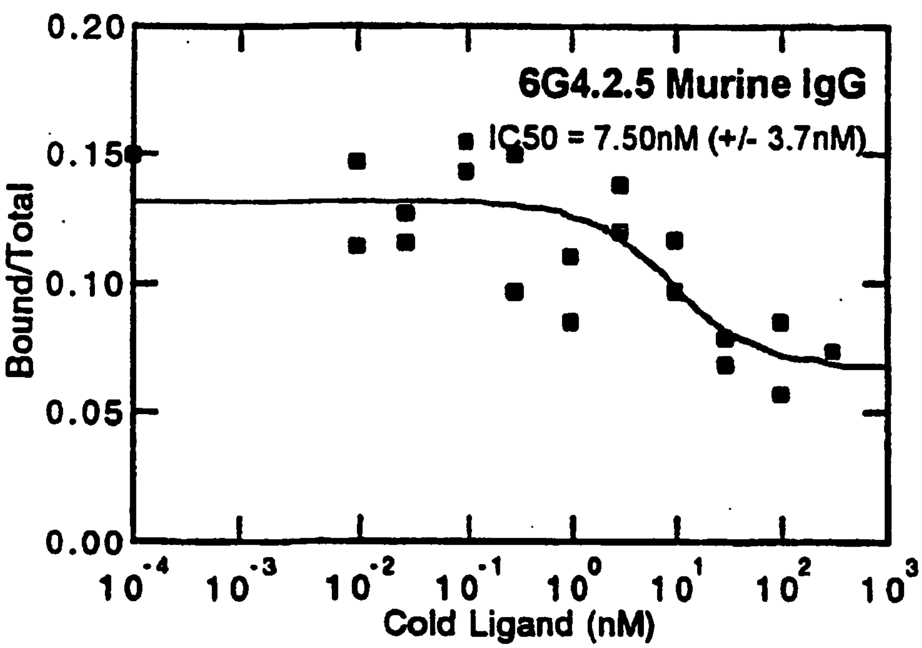


FIG. 49B

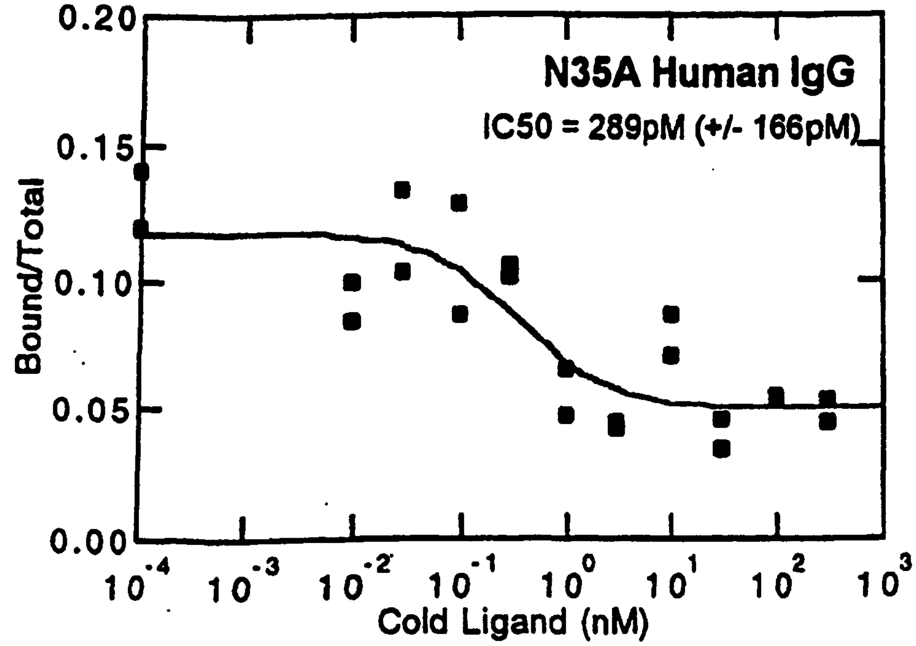


FIG. 49C

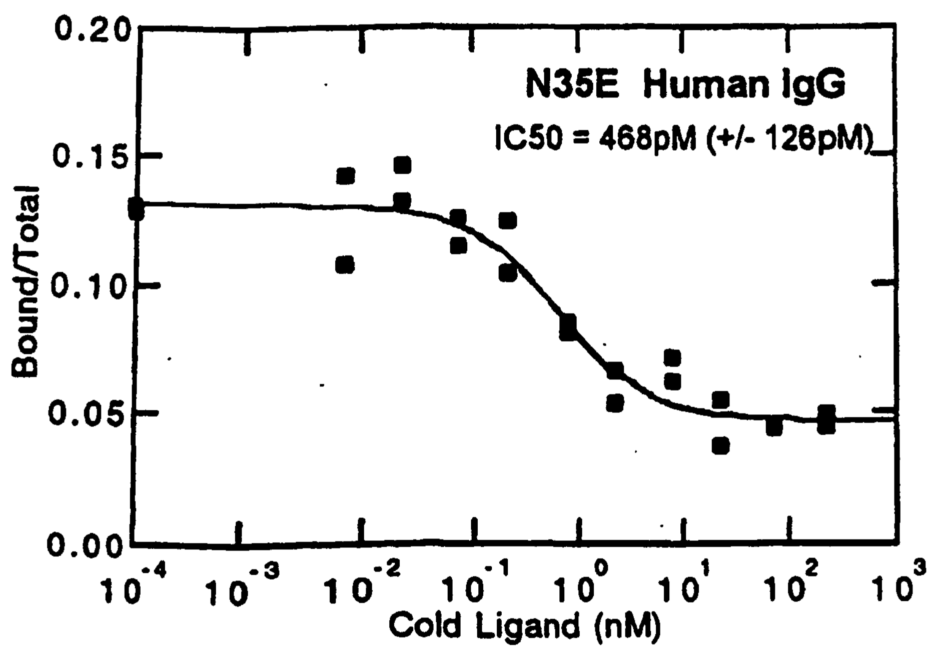


FIG. 49D

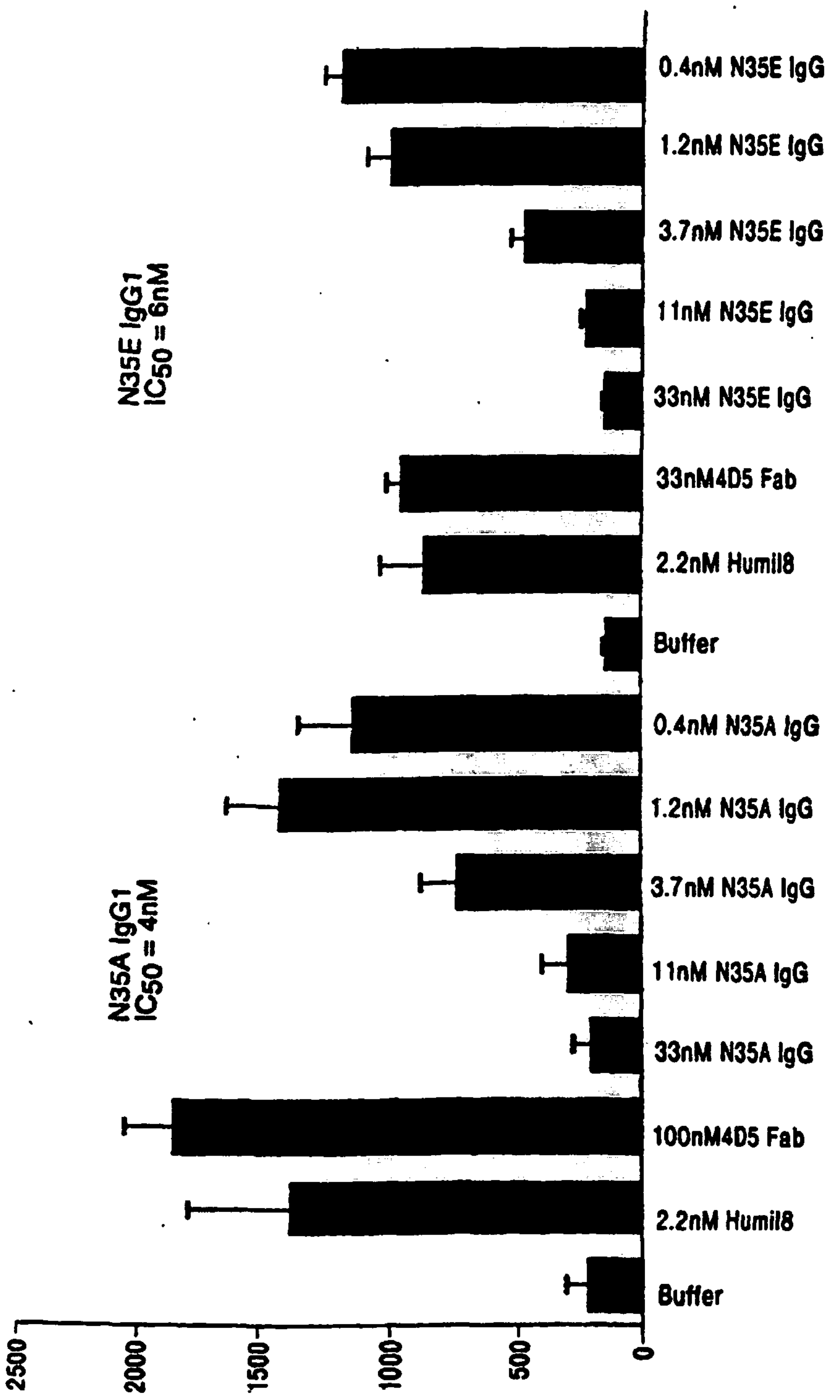

2500
2000
1500
1000
500
0
Buffer
2.2nM Humil8
100nM4D5 Fab
33nM N35A IgG
11nM N35A IgG
3.7nM N35A IgG
1.2nM N35A IgG
0.4nM N35A IgG
Buffer
2.2nM Humil8
33nM4D5 Fab
33nM N35E IgG
11nM N35E IgG
3.7nM N35E IgG
1.2nM N35E IgG
0.4nM N35E IgG
N35A IgG1
IC50 = 4nM
N35E IgG1
IC50 = 6nM

FIG. 50A

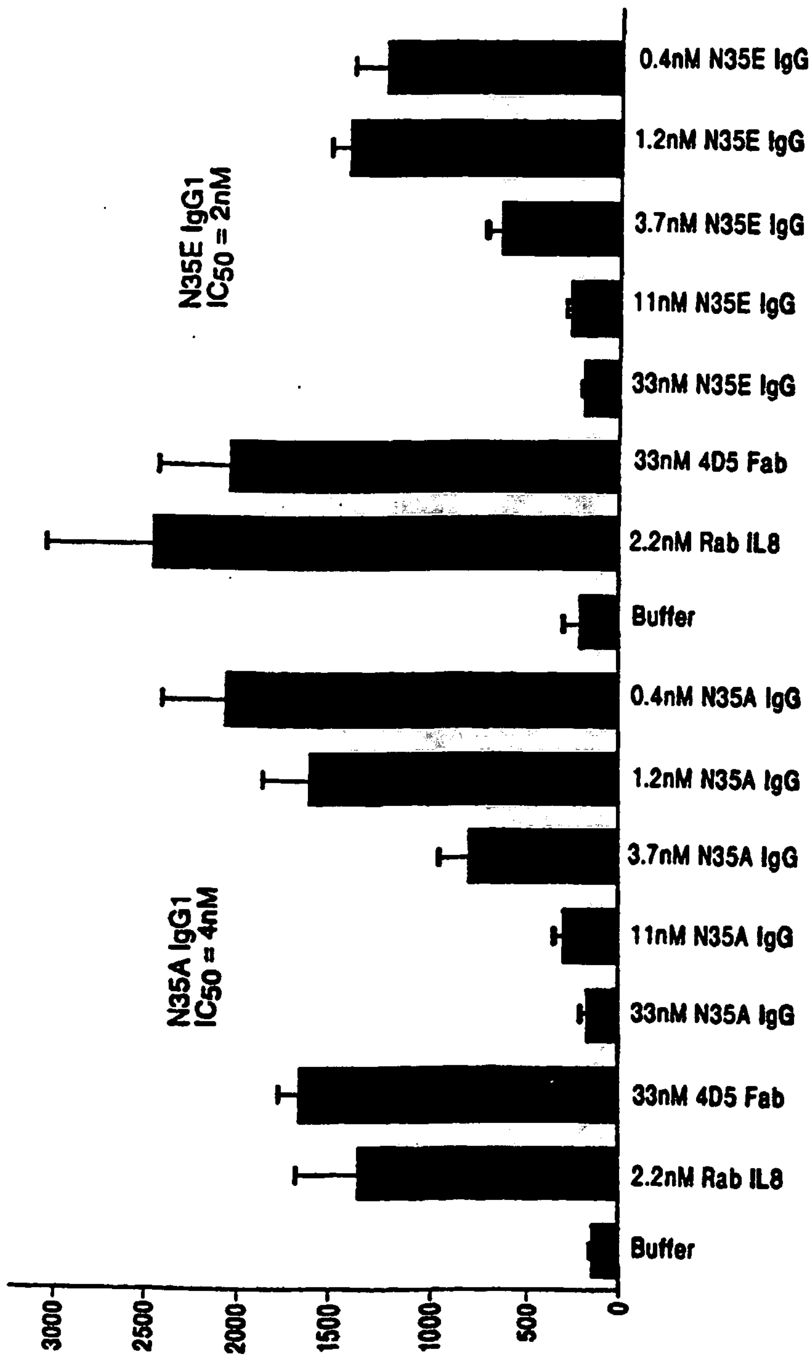
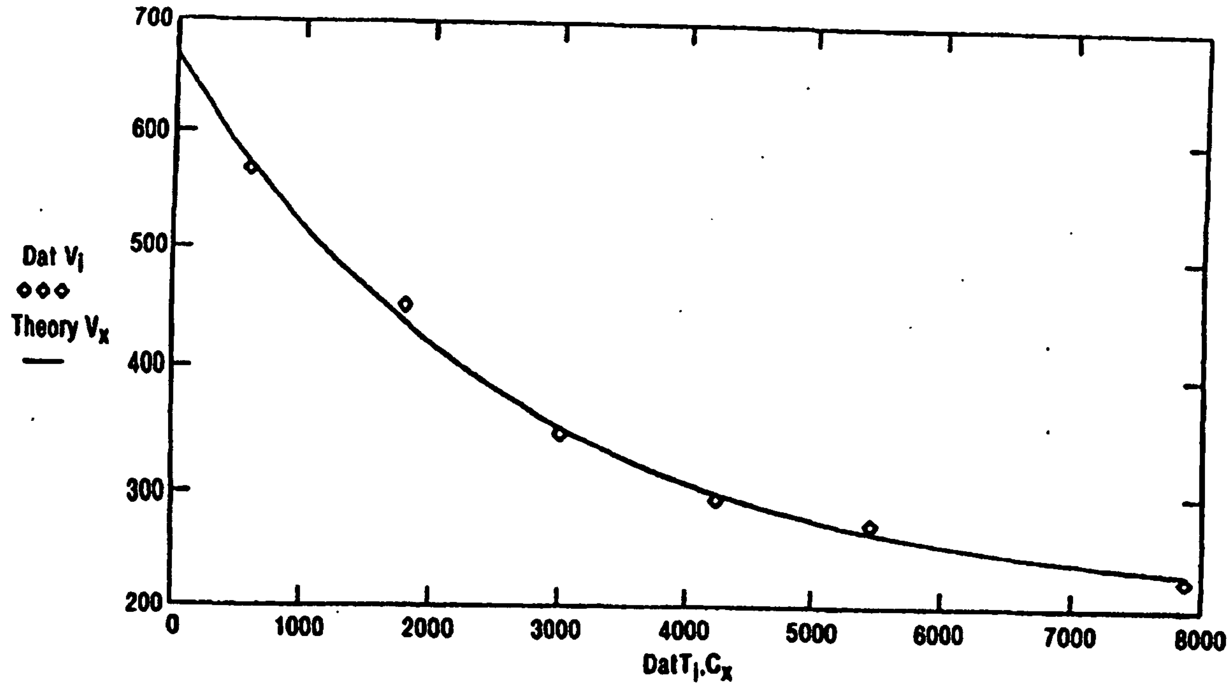

N35E IgG1
IC50 = 2nM
N35A IgG1
IC50 = 4nM
0.4nM N35E IgG
1.2nM N35E IgG
3.7nM N35E IgG
11nM N35E IgG
33nM N35E IgG
33nM 4D5 Fab
2.2nM Rab IL8
Buffer
0.4nM N35A IgG
1.2nM N35A IgG
3.7nM N35A IgG
11nM N35A IgG
33nM N35A IgG
33nM 4D5 Fab
2.2nM Rab IL8
Buffer
3000
2500
2000
1500
1000
500
0

FIG. 50B



Representative Conc versus Time Plot. Shown is the kinetic data for 6G4V11N35A.IgG1

SAMPLE	ka	kd	Kd
Murine 6G4.2.5 IgG2a	8.3×10^5	2.9×10^{-4}	350pM
6G4V11N35A-IgG1	8.7×10^5	7.7×10^{-5}	88pM
6G4V11N35E-IgG1	3.0×10^6	1.4×10^{-4}	49pM

FIG. 51

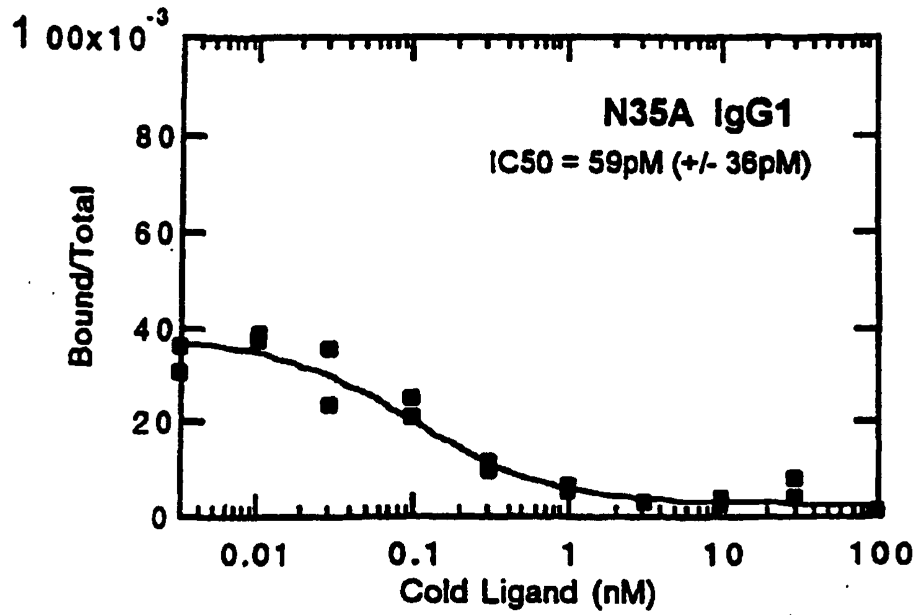


FIG. 52A

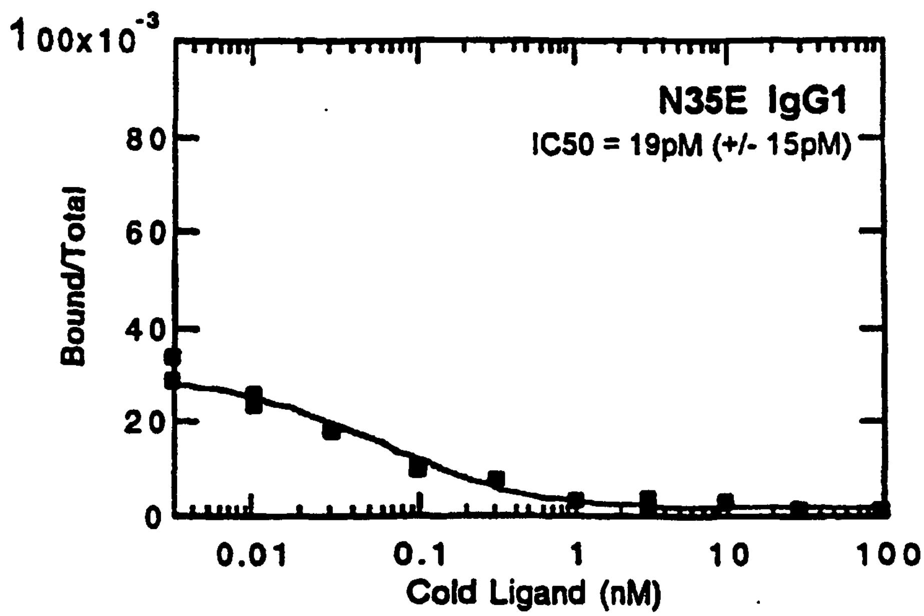


FIG. 52B

```
781  AAAACGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT TCTTCTTGCA
     TTTTCCCATA GATCTCCAAC TCCACTAAAA TACTTTTTCT TATAGCGTAA AGAAGAACGT
-1                                    M  K  K  N   I  A  F   L  L  A

841  TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG AGGTTCAGCT AGTGCAGTCT
     AGATACAAGC AAAAAAGATA ACGATGTTTG CGCATGCGAC TCCAAGTCGA TCACGTCAGA
-11  S  M  F  V   F  S  I   A  T  N   A  Y  A  E   V  Q  L   V  Q  S

901  GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA CTCCGTTTGT CCTGTGCAGC TTCTGGCTAC
     CCGCCACCGG ACCACGTCGG TCCCCCGAGT GAGGCAAACA GGACACGTCG AAGACCGATG
8    G  G  G  L   V  Q  P   G  G  S   L  R  L  S   C  A  A   S  G  Y

961  TCCTTCTCGA GTCACTATAT GCACTGGGTC CGTCAGGCCC CGGGTAAGGG CCTGGAATGG
     AGGAAGAGCT CAGTGATATA CGTGACCCAG GCAGTCCGGG GCCCATTCCC GGACCTTACC
28   S  F  S  S   H  Y  M   H  W  V   R  Q  A  P   G  K  G   L  E  W

1021 GTTGGATATA TTGATCCTTC CAATGGTGAA ACTACGTATA ATCAAAAGTT CAAGGGCCGT
     CAACCTATAT AACTAGGAAG GTTACCACTT TGATGCATAT TAGTTTTCAA GTTCCCGGCA
48   V  G  Y  I   D  P  S   N  G  E   T  T  Y  N   Q  K  F   K  G  R

1081 TTCACTTTAT CTCGCGACAA CTCCAAAAAC ACAGCATACC TGCAGATGAA CAGCCTGCGT
     AAGTGAAATA GAGCGCTGTT GAGGTTTTTG TGTCGTATGG ACGTCTACTT GTCGGACGCA
68   F  T  L  S   R  D  N   S  K  N   T  A  Y  L   Q  M  N   S  L  R

1141 GCTGAGGACA CTGCCGTCTA TTACTGTGCA AGAGGGGATT ATCGCTACAA TGGTGACTGG
     CGACTCCTGT GACGGCAGAT AATGACACGT TCTCCCCTAA TAGCGATGTT ACCACTGACC
88   A  E  D  T   A  V  Y   Y  C  A   R  G  D  Y   R  Y  N   G  D  W

1201 TTCTTCGACG TCTGGGGTCA AGGAACCCTG GTCACCGTCT CCTCGGCCTC CACCAAGGGC
     AAGAAGCTGC AGACCCCAGT TCCTTGGGAC CAGTGGCAGA GGAGCCGGAG GTGGTTCCCG
108  F  F  D  V   W  G  Q   G  T  L   V  T  V  S   S  A  S   T  K  G

1261 CCATCGGTCT TCCCCCTGGC ACCCTCCTCC AAGAGCACCT CTGGGGGCAC AGCGGCCCTG
     GGTAGCCAGA AGGGGGACCG TGGGAGGAGG TTCTCGTGGA GACCCCCGTG TCGCCGGGAC
128  P  S  V  F   P  L  A   P  S  S   K  S  T  S   G  G  T   A  A  L

1321 GGCTGCCTGG TCAAGGACTA CTTCCCCGAA CCGGTGACGG TGTCGTGGAA CTCAGGCGCC
     CCGACGGACC AGTTCCTGAT GAAGGGGCTT GGCCACTGCC ACAGCACCTT GAGTCCGCGG
148  G  C  L  V   K  D  Y   F  P  E   P  V  T  V   S  W  N   S  G  A

1381 CTGACCAGCG GCGTGCACAC CTTCCCGGCT GTCCTACAGT CCTCAGGACT CTACTCCCTC
     GACTGGTCGC CGCACGTGTG GAAGGGCCGA CAGGATGTCA GGAGTCCTGA GATGAGGGAG
168  L  T  S  G   V  H  T   F  P  A   V  L  Q  S   S  G  L   Y  S  L

1441 AGCAGCGTGG TGACCGTGCC CTCCAGCAGC TTGGGCACCC AGACCTACAT CTGCAACGTG
     TCGTCGCACC ACTGGCACGG GAGGTCGTCG AACCCGTGGG TCTGGATGTA GACGTTGCAC
188  S  S  V  V   T  V  P   S  S  S   L  G  T  Q   T  Y  I   C  N  V

1501 AATCACAAGC CCAGCAACAC CAAGGTCGAC AAGAAAGTTG AGCCCAAATC TTGTGACAAA
     TTAGTGTTCG GGTCGTTGTG GTTCCAGCTG TTCTTTCAAC TCGGGTTTAG AACACTGTTT
208  N  H  K  P   S  N  T   K  V  D   K  K  V  E   P  K  S   C  D  K

1561 ACTCACACAT GCCCGCCGTGA
     TGAGTGTGTA CGGGCGGCACT
228  T  H  T  C   P  P  O
```

FIG. 53

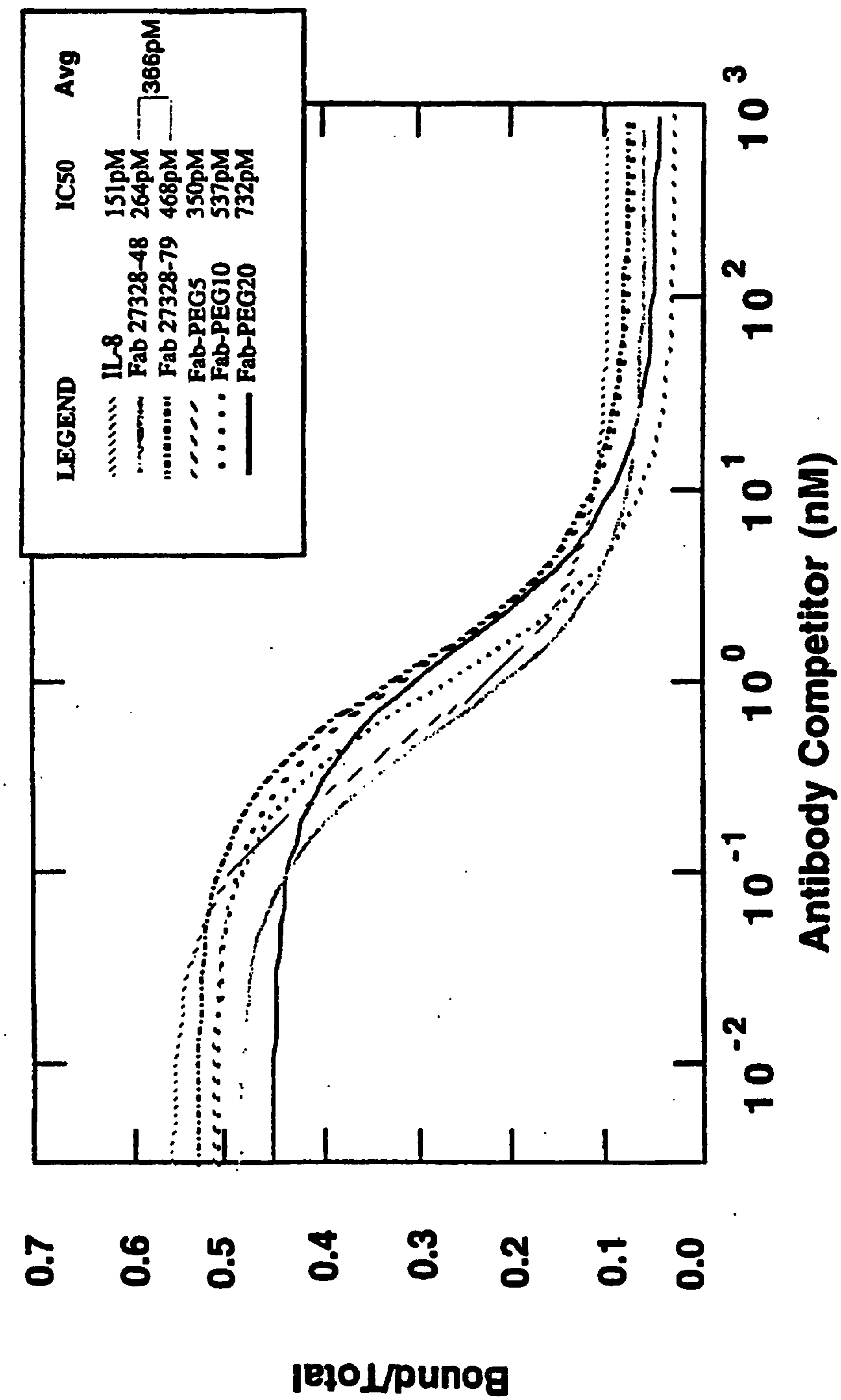

LEGEND
IC50
Avg
IL-8
151pM
Fab 27328-48
264pM
Fab 27328-79
468pM
366pM
Fab-PEG5
350pM
Fab-PEG10
537pM
Fab-PEG20
732pM
Bound/Total
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Antibody Competitor (nM)

FIG. 54A

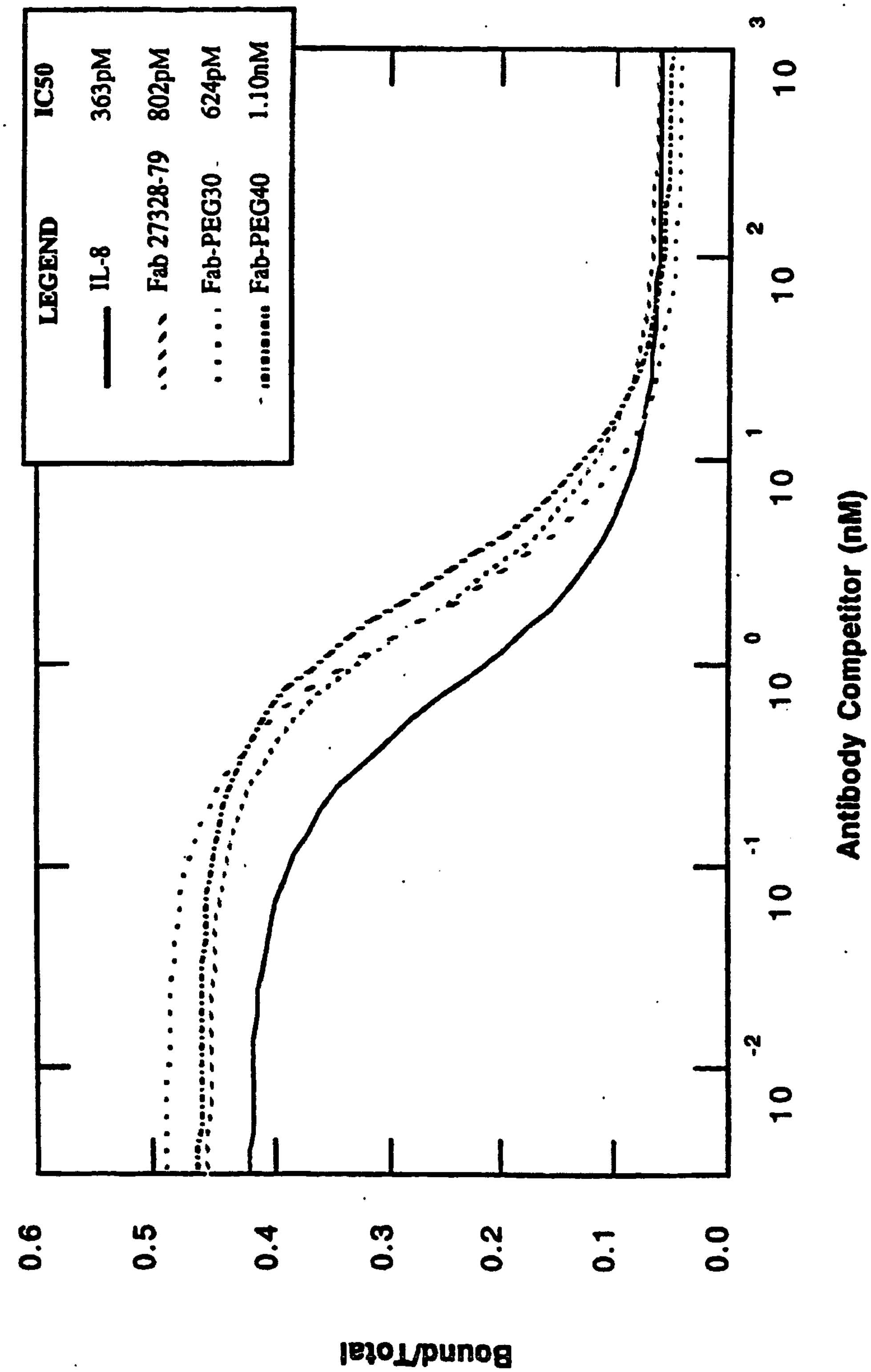

LEGEND
IL-8
Fab 27328-79
Fab-PEG30
Fab-PEG40
IC50
363pM
802pM
624pM
1.10nM
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Bound/Total
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Antibody Competitor (nM)

FIG. 54B

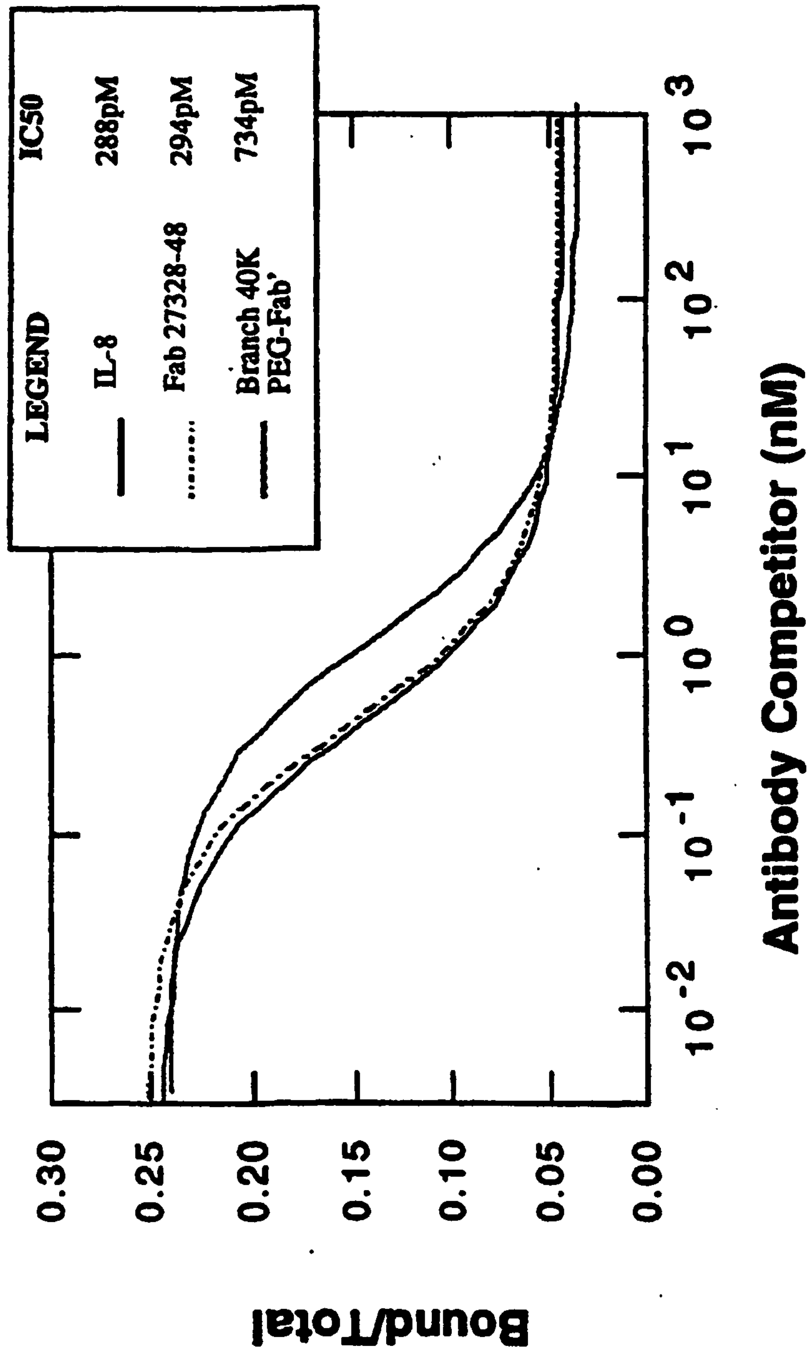

LEGEND
IC50
IL-8
288pM
Fab 27328-48
294pM
Branch 40K PEG-Fab'
734pM
Bound/Total
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Antibody Competitor (nM)

FIG. 54C

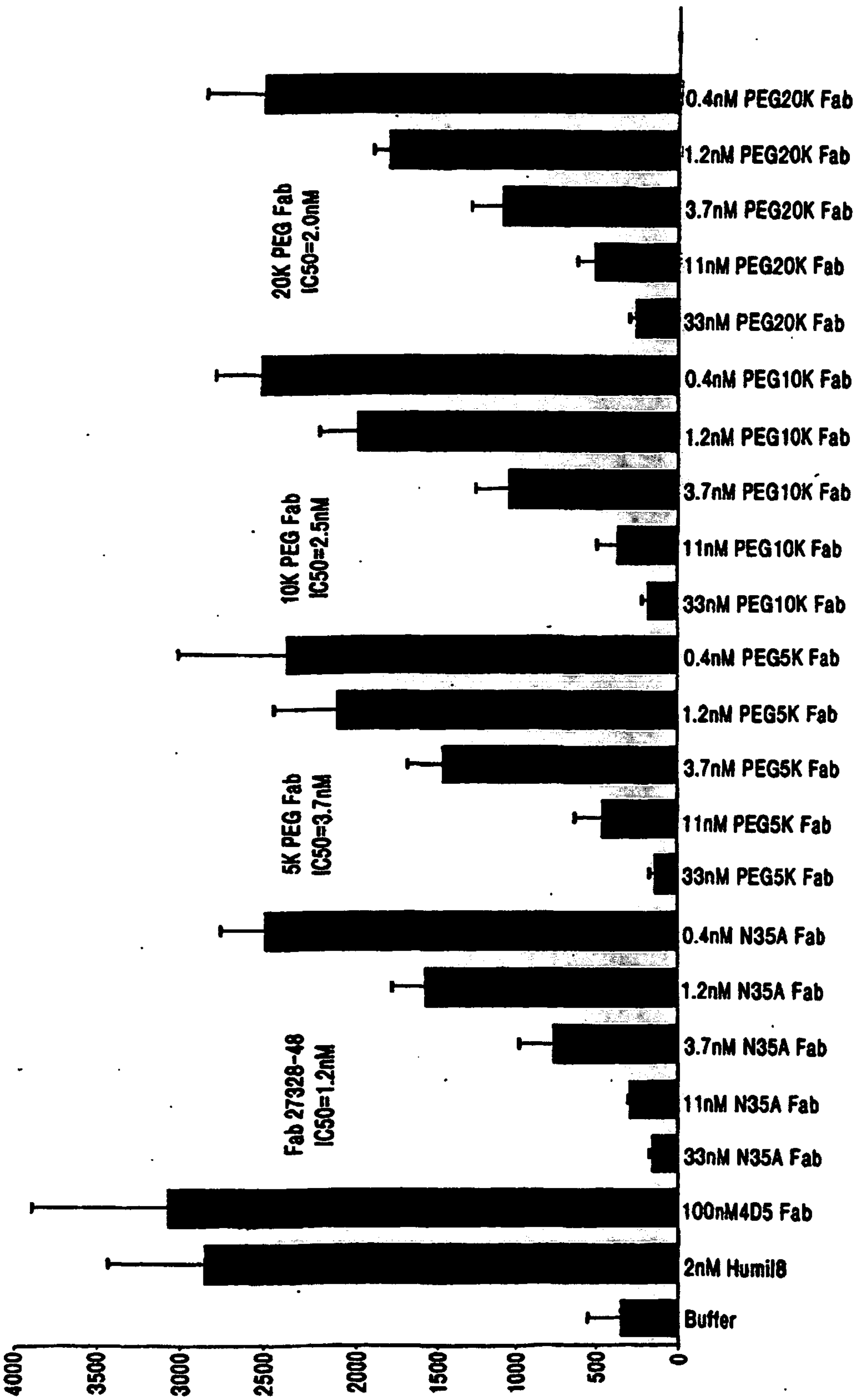

0.4nM PEG20K Fab
1.2nM PEG20K Fab
3.7nM PEG20K Fab
11nM PEG20K Fab
33nM PEG20K Fab
0.4nM PEG10K Fab
1.2nM PEG10K Fab
3.7nM PEG10K Fab
11nM PEG10K Fab
33nM PEG10K Fab
0.4nM PEG5K Fab
1.2nM PEG5K Fab
3.7nM PEG5K Fab
11nM PEG5K Fab
33nM PEG5K Fab
0.4nM N35A Fab
1.2nM N35A Fab
3.7nM N35A Fab
11nM N35A Fab
33nM N35A Fab
100nM4D5 Fab
2nM Humil8
Buffer
20K PEG Fab
IC50=2.0nM
10K PEG Fab
IC50=2.5nM
5K PEG Fab
IC50=3.7nM
Fab 27328-48
IC50=1.2nM
0
500
1000
1500
2000
2500
3000
3500
4000

FIG. 55A

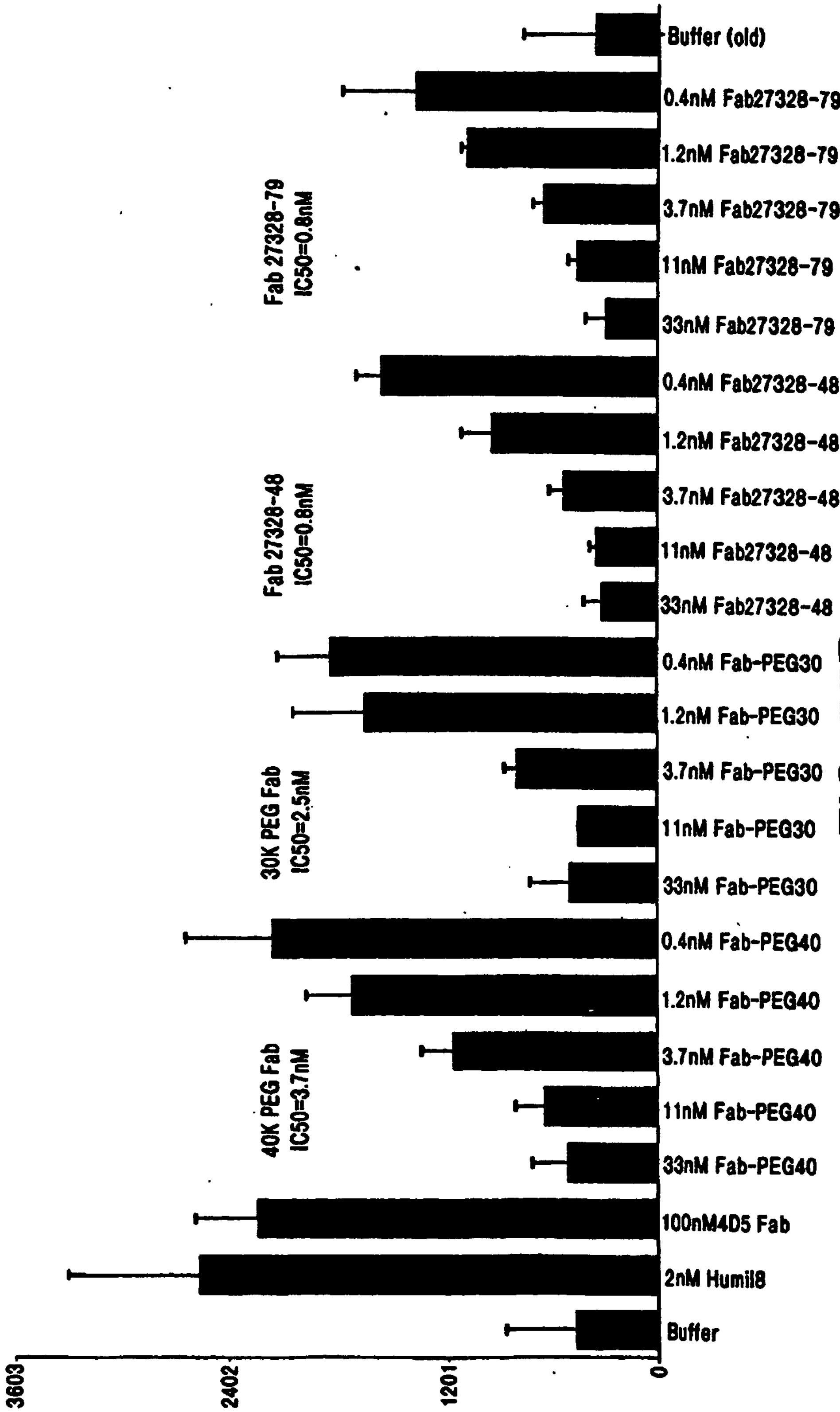

Buffer (old)
0.4nM Fab27328-79
1.2nM Fab27328-79
3.7nM Fab27328-79
11nM Fab27328-79
33nM Fab27328-79
0.4nM Fab27328-48
1.2nM Fab27328-48
3.7nM Fab27328-48
11nM Fab27328-48
33nM Fab27328-48
0.4nM Fab-PEG30
1.2nM Fab-PEG30
3.7nM Fab-PEG30
11nM Fab-PEG30
33nM Fab-PEG30
0.4nM Fab-PEG40
1.2nM Fab-PEG40
3.7nM Fab-PEG40
11nM Fab-PEG40
33nM Fab-PEG40
100nM4D5 Fab
2nM Humil8
Buffer
Fab 27328-79
IC50=0.8nM
Fab 27328-48
IC50=0.8nM
30K PEG Fab
IC50=2.5nM
40K PEG Fab
IC50=3.7nM
3603
2402
1201
0

FIG. 55B

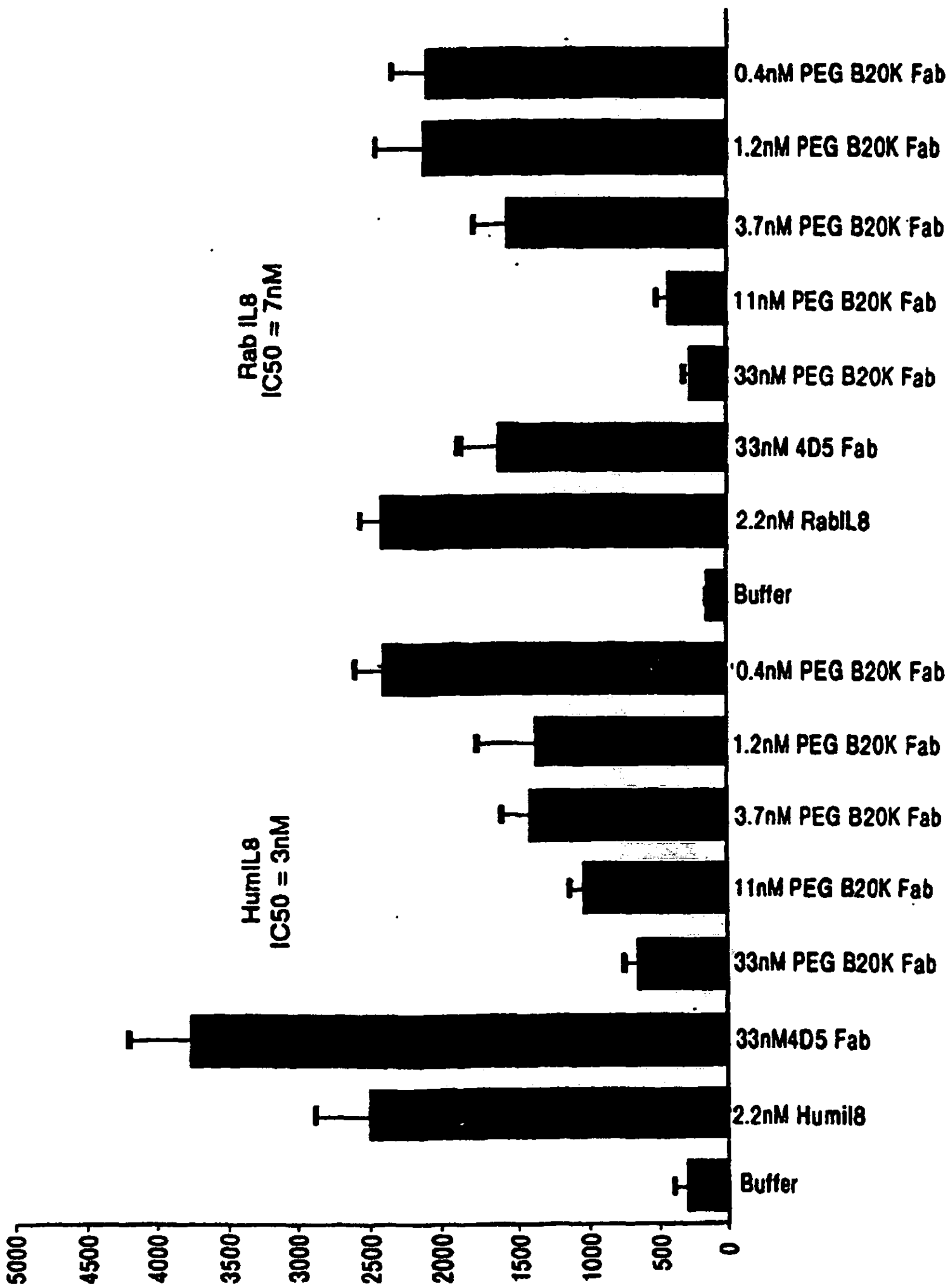

Rab IL8
IC50 = 7nM
HumIL8
IC50 = 3nM
0.4nM PEG B20K Fab
1.2nM PEG B20K Fab
3.7nM PEG B20K Fab
11nM PEG B20K Fab
33nM PEG B20K Fab
33nM 4D5 Fab
2.2nM RabIL8
Buffer
0.4nM PEG B20K Fab
1.2nM PEG B20K Fab
3.7nM PEG B20K Fab
11nM PEG B20K Fab
33nM PEG B20K Fab
33nM4D5 Fab
2.2nM Humil8
Buffer
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000

FIG. 55C

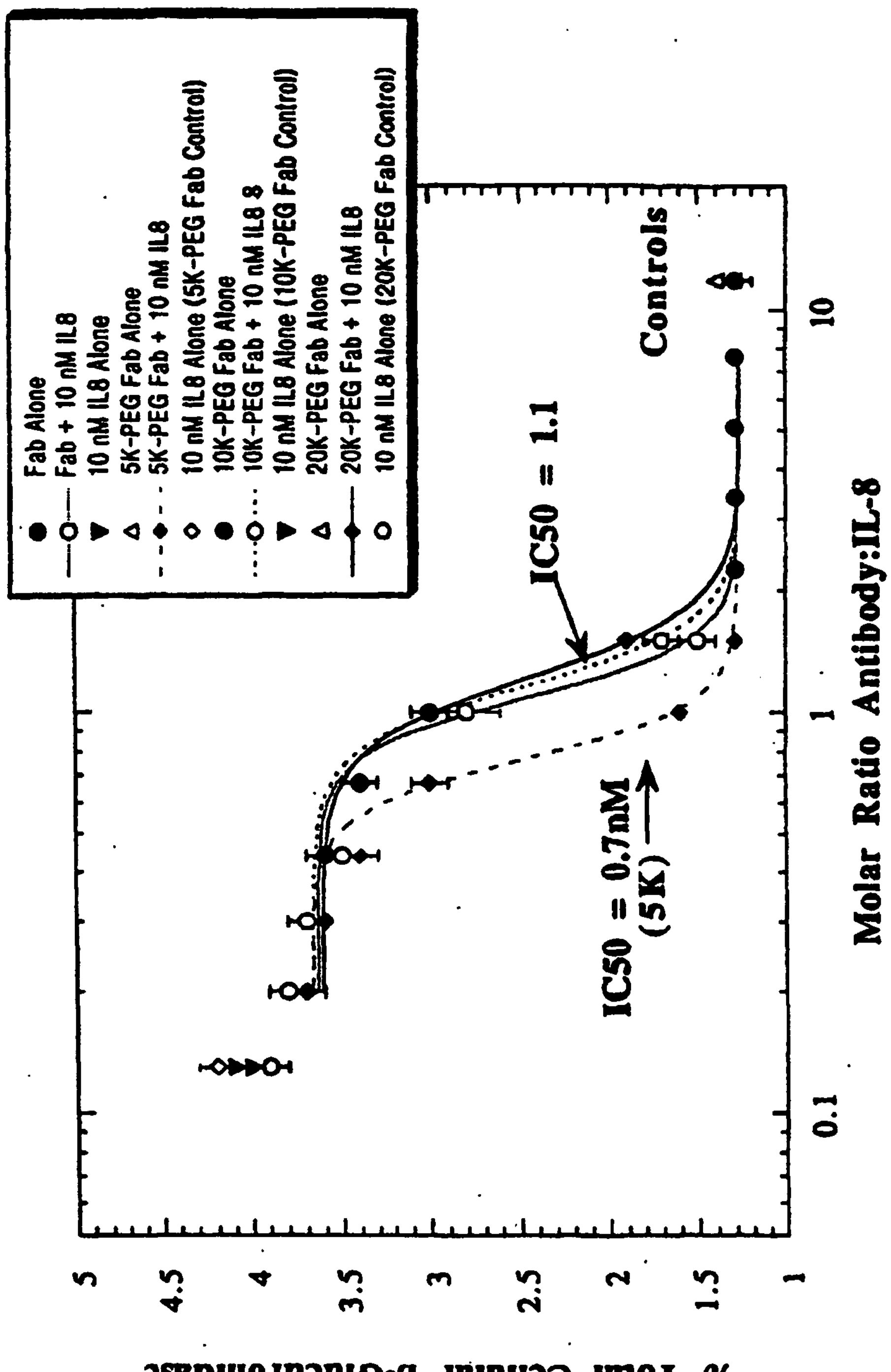

Fab Alone
Fab + 10 nM IL8
10 nM IL8 Alone
5K-PEG Fab Alone
5K-PEG Fab + 10 nM IL8
10 nM IL8 Alone (5K-PEG Fab Control)
10K-PEG Fab Alone
10K-PEG Fab + 10 nM IL8
10 nM IL8 Alone (10K-PEG Fab Control)
20K-PEG Fab Alone
20K-PEG Fab + 10 nM IL8
10 nM IL8 Alone (20K-PEG Fab Control)
IC50 = 1.1
IC50 = 0.7nM
(5K)
Controls
% Total Cellular ß-Glucuronidase
Molar Ratio Antibody:IL-8
5
4.5
4
3.5
3
2.5
2
1.5
1
0.1
1
10

FIG. 56A

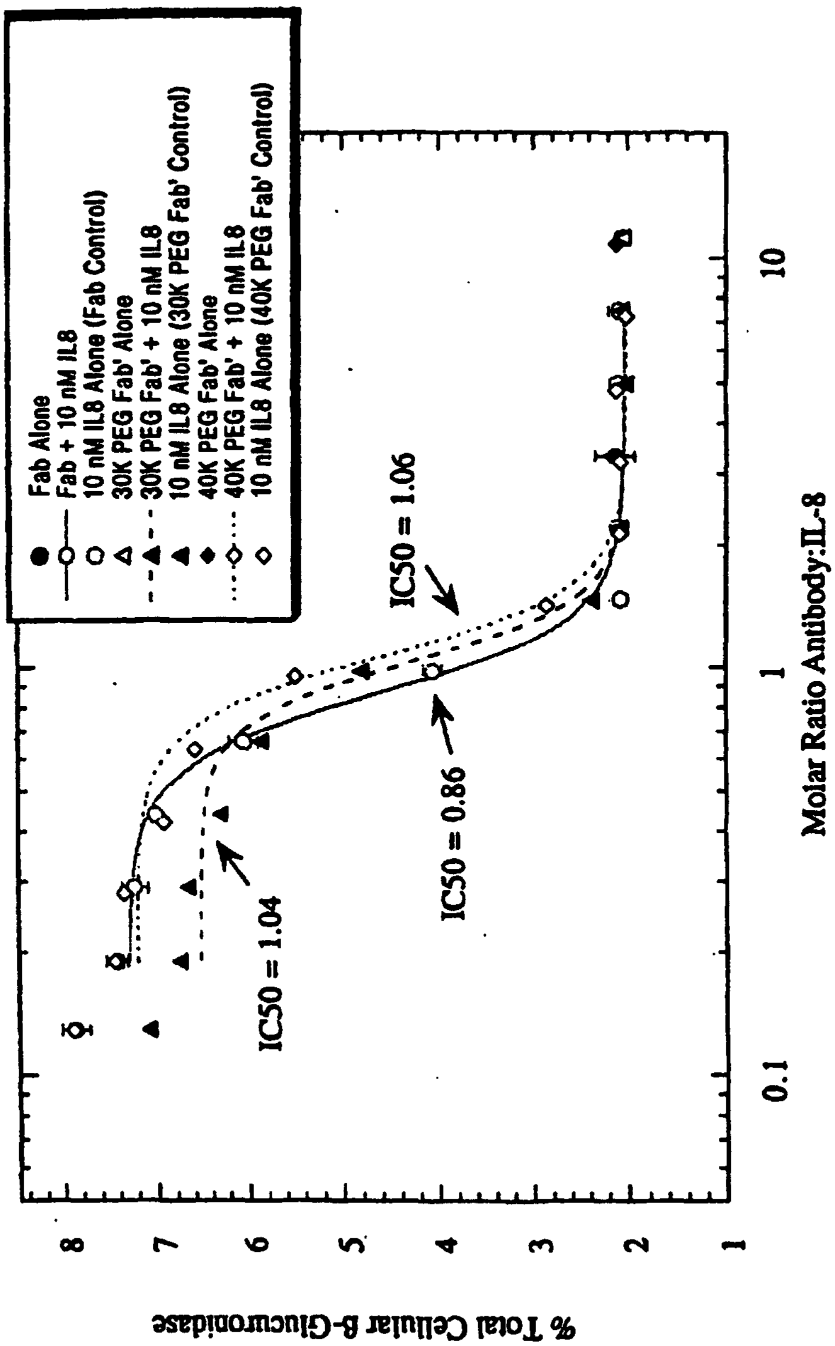

Fab Alone
Fab + 10 nM IL8
10 nM IL8 Alone (Fab Control)
30K PEG Fab' Alone
30K PEG Fab' + 10 nM IL8
10 nM IL8 Alone (30K PEG Fab' Control)
40K PEG Fab' Alone
40K PEG Fab' + 10 nM IL8
10 nM IL8 Alone (40K PEG Fab' Control)
IC50 = 1.06
IC50 = 0.86
IC50 = 1.04
% Total Cellular ß-Glucuronidase
Molar Ratio Antibody:IL-8
0.1
1
10
1
2
3
4
5
6
7
8

FIG. 56B

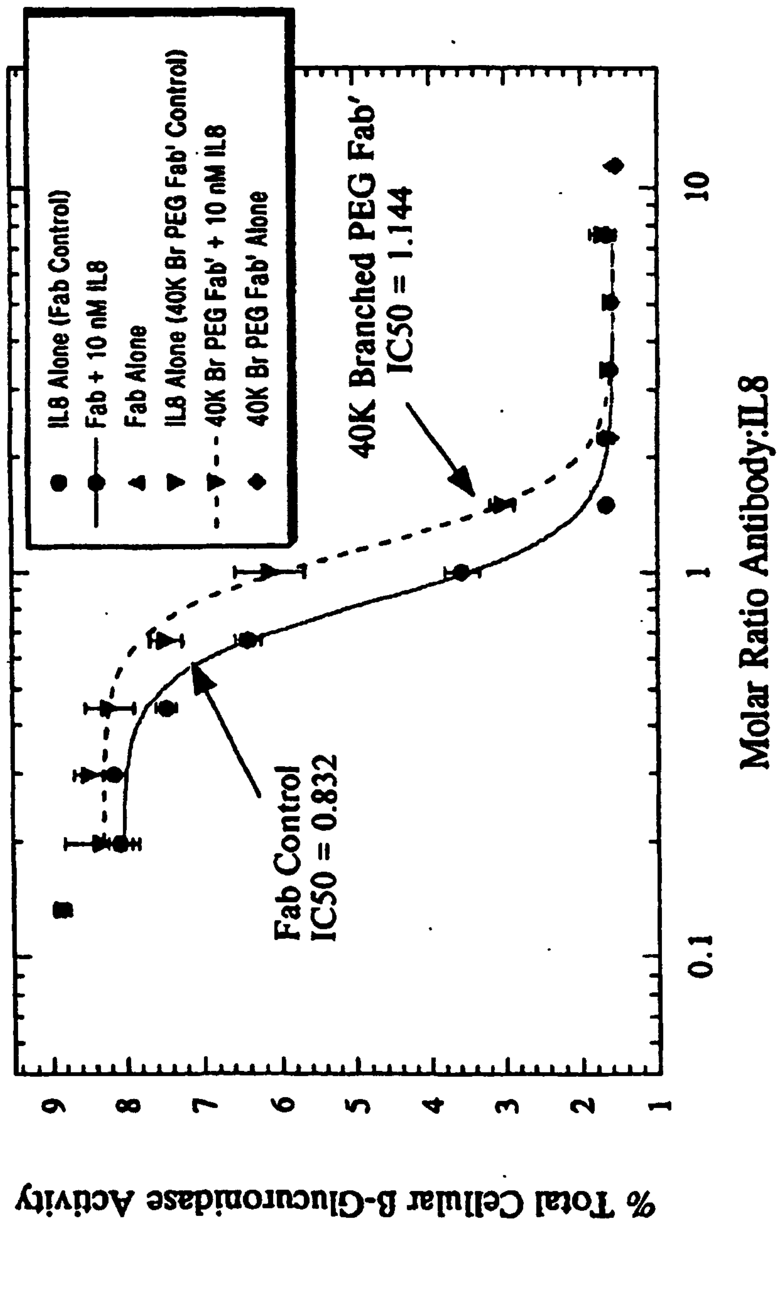

IL8 Alone (Fab Control)
Fab + 10 nM IL8
Fab Alone
IL8 Alone (40K Br PEG Fab' Control)
40K Br PEG Fab' + 10 nM IL8
40K Br PEG Fab' Alone
40K Branched PEG Fab'
IC50 = 1.144
Fab Control
IC50 = 0.832
0.1
1
10
Molar Ratio Antibody:IL8
9
8
7
6
5
4
3
2
1
% Total Cellular ß-Glucuronidase Activity

FIG. 56C

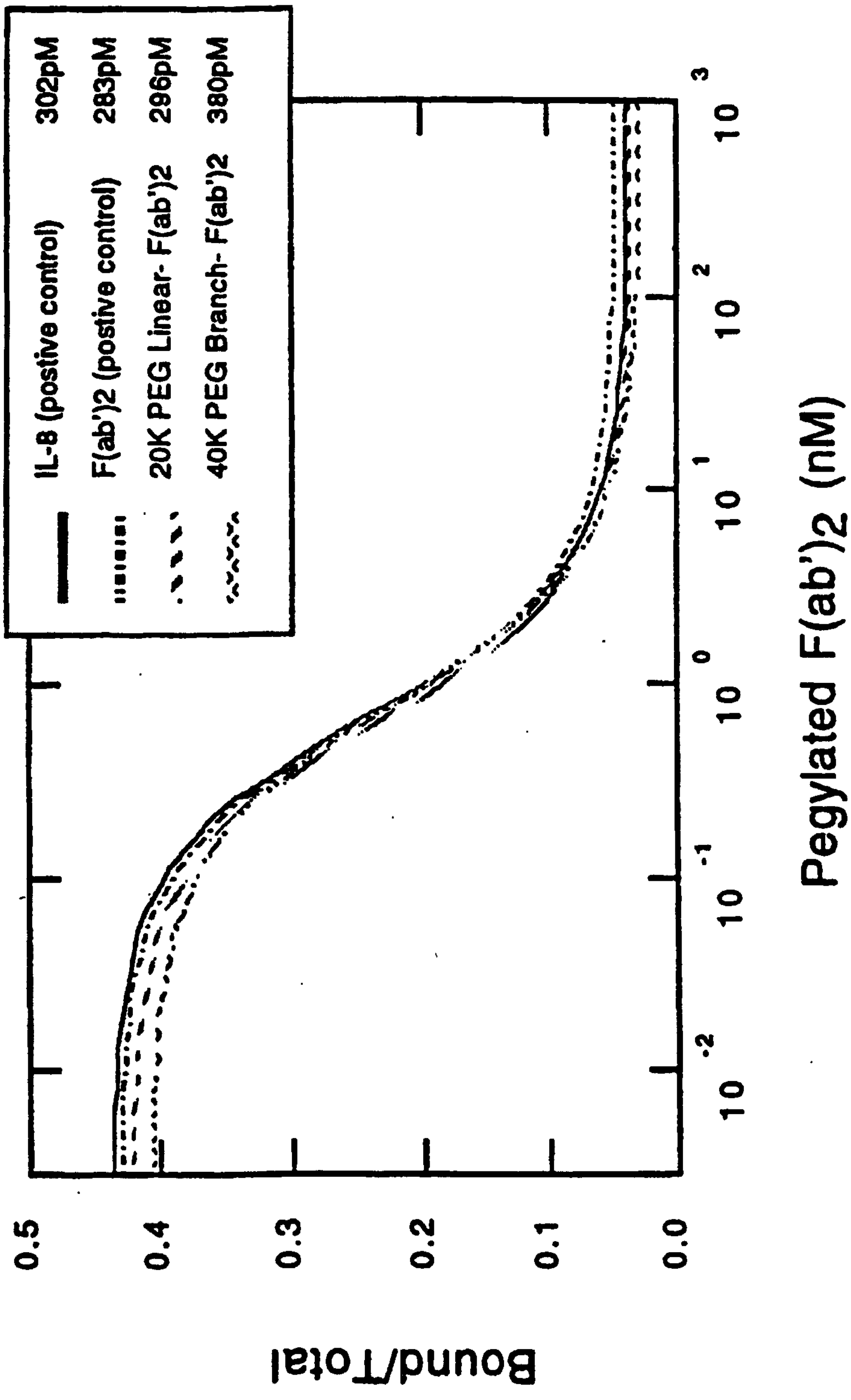

IL-8 (postive control) 302pM
F(ab')2 (postive control) 283pM
20K PEG Linear- F(ab')2 296pM
40K PEG Branch- F(ab')2 380pM
Bound/Total
0.0
0.1
0.2
0.3
0.4
0.5
10^-2
10^-1
10^0
10^1
10^2
10^3
Pegylated F(ab')2 (nM)

FIG. 57A

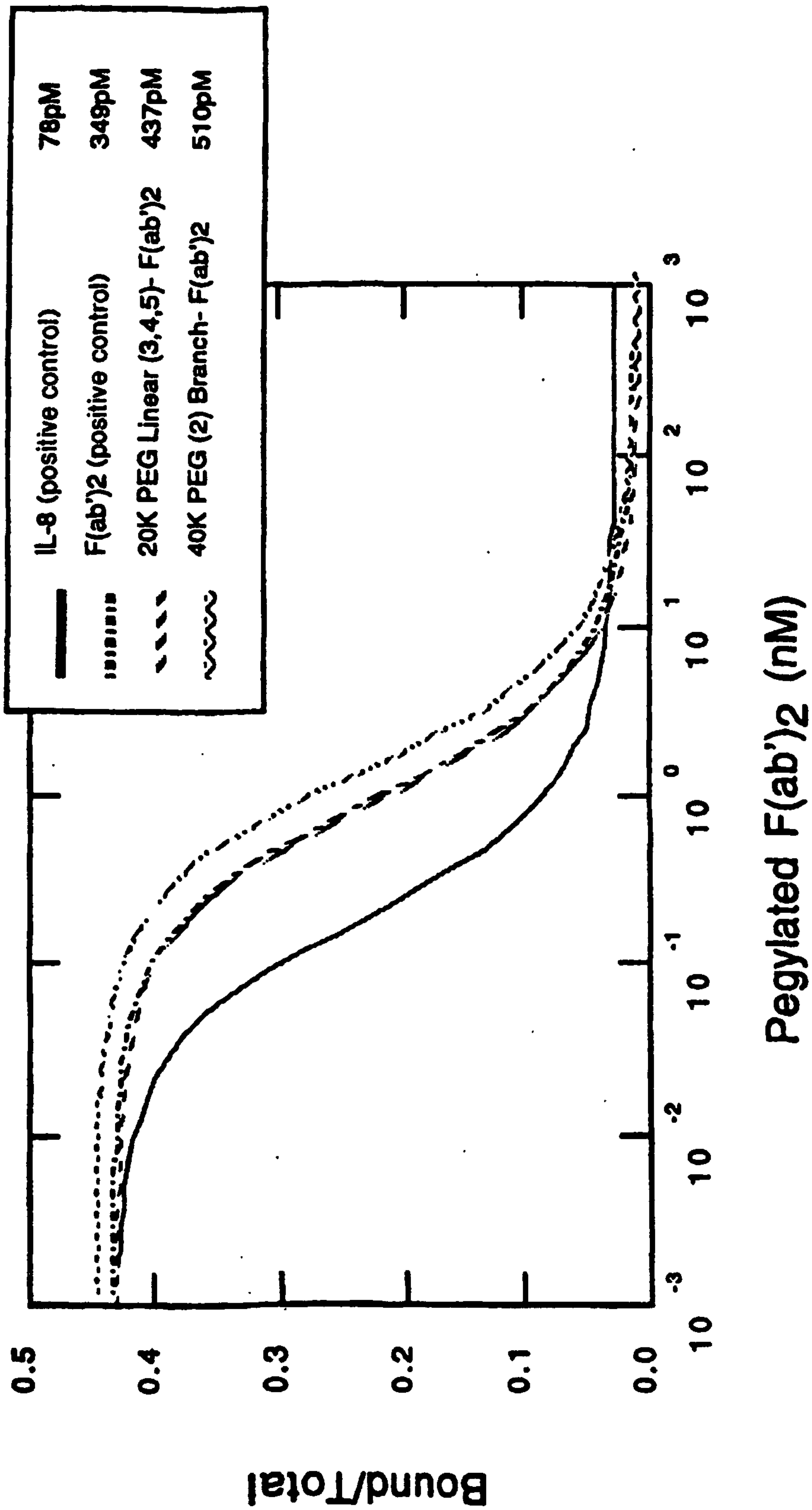

IL-8 (positive control) 78pM
F(ab')2 (positive control) 349pM
20K PEG Linear (3,4,5)- F(ab')2 437pM
40K PEG (2) Branch- F(ab')2 510pM
Bound/Total
0.5
0.4
0.3
0.2
0.1
0.0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Pegylated F(ab')$_2$ (nM)

FIG. 57B

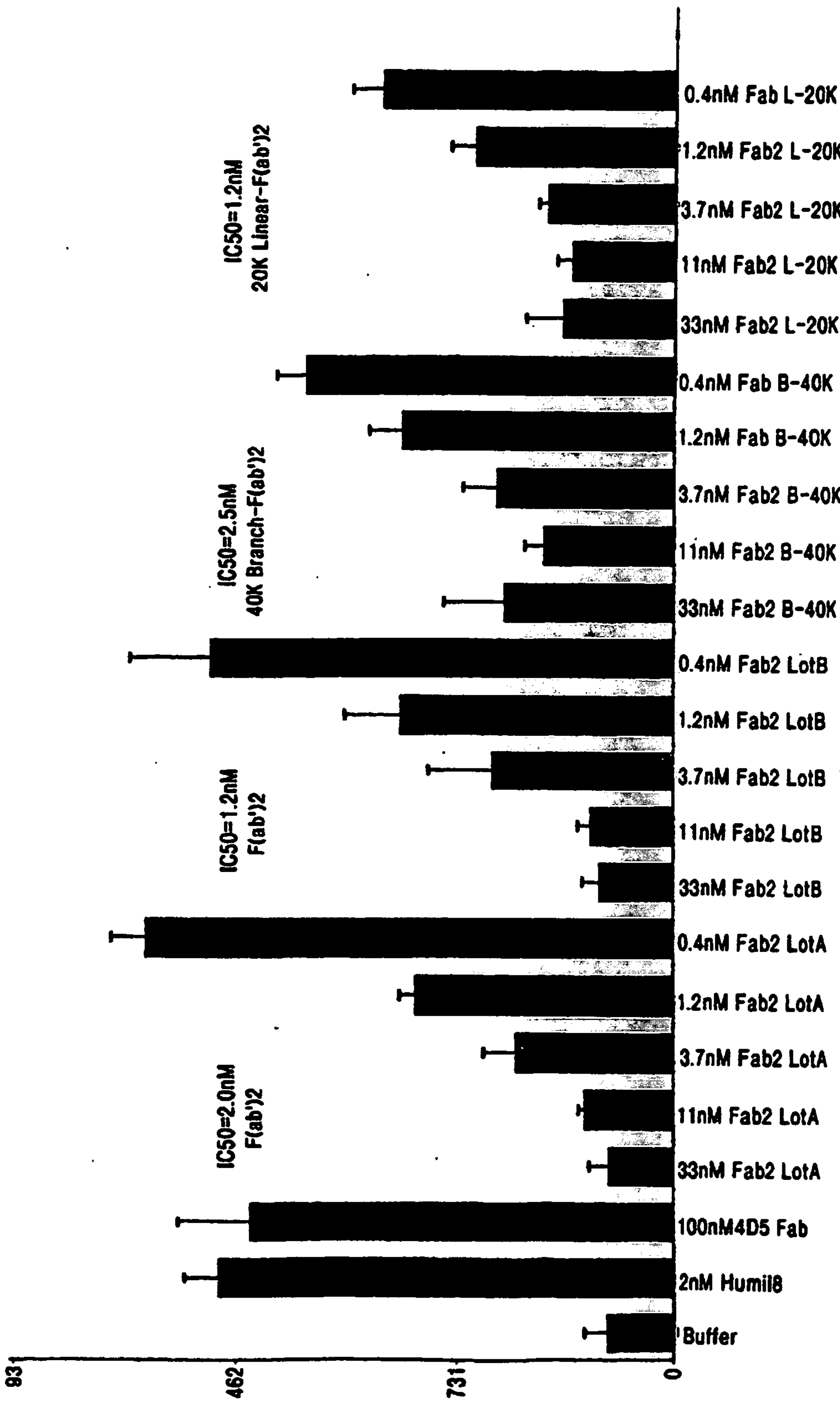

IC50=1.2nM
20K Linear-F(ab')2
IC50=2.5nM
40K Branch-F(ab')2
IC50=1.2nM
F(ab')2
IC50=2.0nM
F(ab')2
0.4nM Fab L-20K
1.2nM Fab2 L-20K
3.7nM Fab2 L-20K
11nM Fab2 L-20K
33nM Fab2 L-20K
0.4nM Fab B-40K
1.2nM Fab B-40K
3.7nM Fab2 B-40K
11nM Fab2 B-40K
33nM Fab2 B-40K
0.4nM Fab2 LotB
1.2nM Fab2 LotB
3.7nM Fab2 LotB
11nM Fab2 LotB
33nM Fab2 LotB
0.4nM Fab2 LotA
1.2nM Fab2 LotA
3.7nM Fab2 LotA
11nM Fab2 LotA
33nM Fab2 LotA
100nM4D5 Fab
2nM Humil8
Buffer
93
462
731
0

FIG. 58A

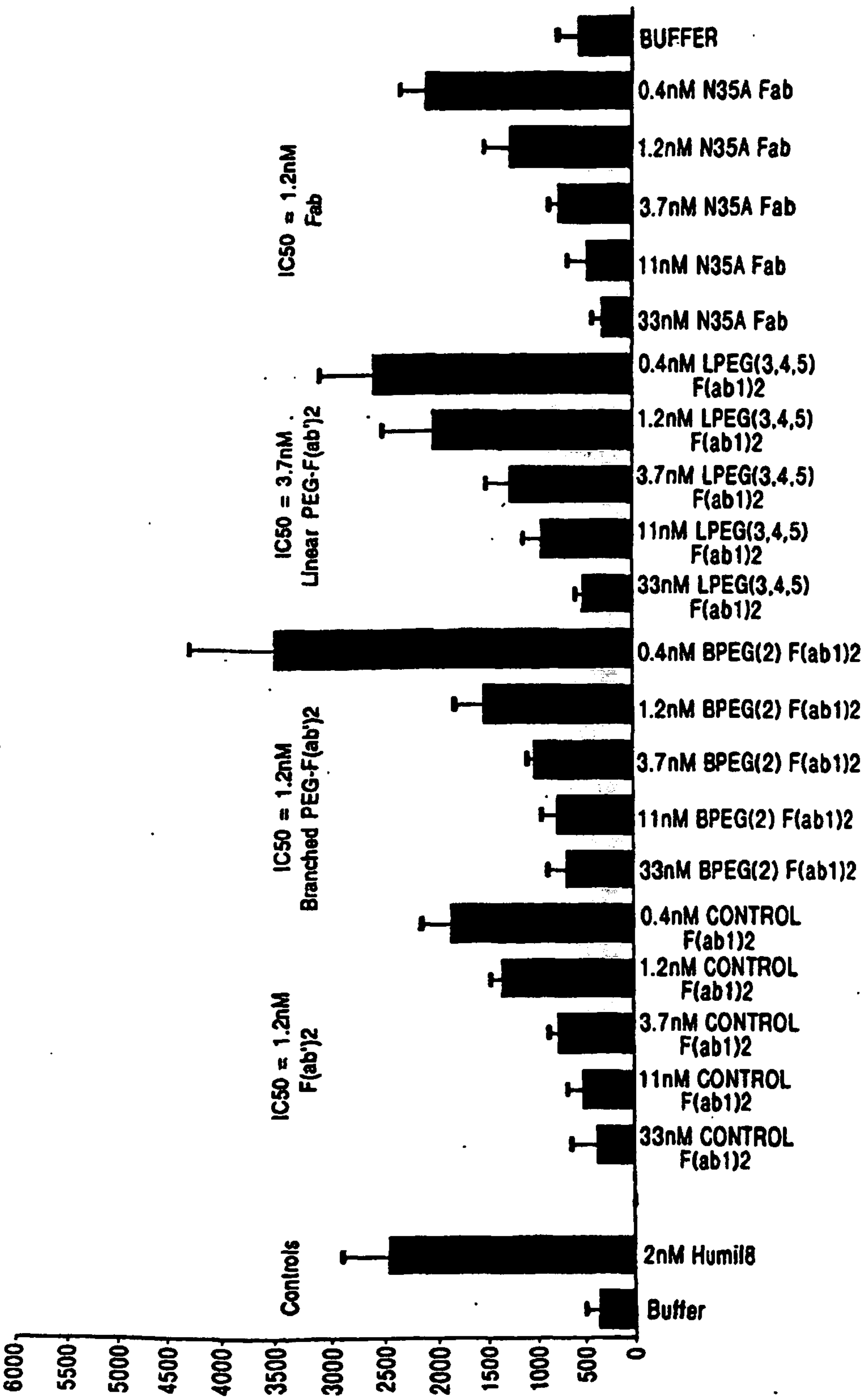

BUFFER
0.4nM N35A Fab
1.2nM N35A Fab
3.7nM N35A Fab
11nM N35A Fab
33nM N35A Fab
0.4nM LPEG(3,4,5) F(ab1)2
1.2nM LPEG(3,4,5) F(ab1)2
3.7nM LPEG(3,4,5) F(ab1)2
11nM LPEG(3,4,5) F(ab1)2
33nM LPEG(3,4,5) F(ab1)2
0.4nM BPEG(2) F(ab1)2
1.2nM BPEG(2) F(ab1)2
3.7nM BPEG(2) F(ab1)2
11nM BPEG(2) F(ab1)2
33nM BPEG(2) F(ab1)2
0.4nM CONTROL F(ab1)2
1.2nM CONTROL F(ab1)2
3.7nM CONTROL F(ab1)2
11nM CONTROL F(ab1)2
33nM CONTROL F(ab1)2
2nM Humil8
Buffer
IC50 = 1.2nM Fab
IC50 = 3.7nM Linear PEG-F(ab')2
IC50 = 1.2nM Branched PEG-F(ab')2
IC50 = 1.2nM F(ab')2
Controls
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0

FIG. 58B

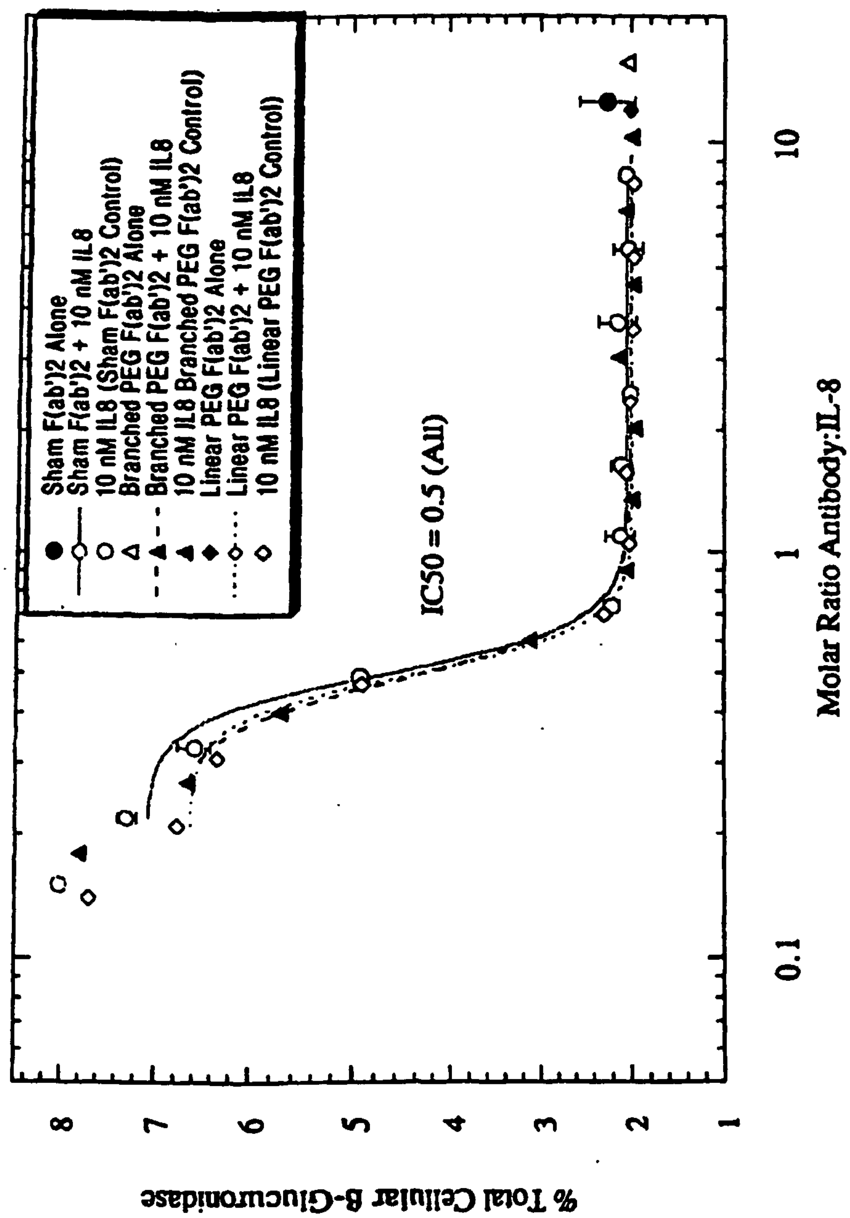
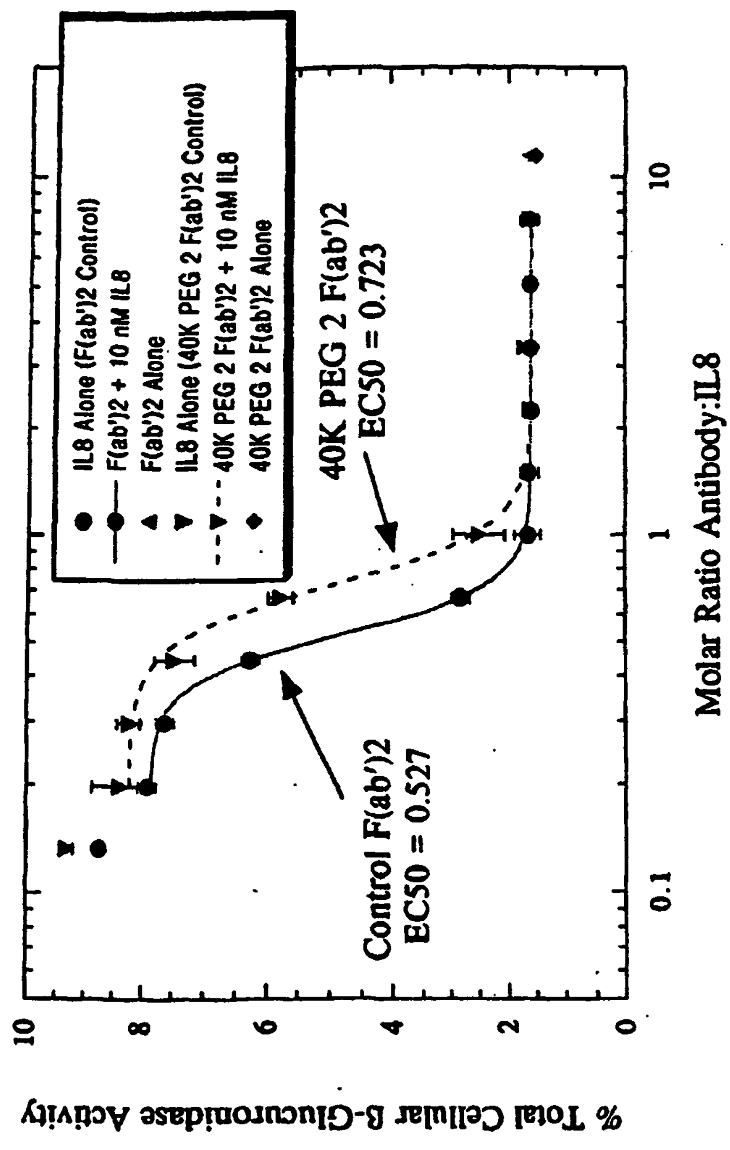

Sham F(ab')2 Alone
Sham F(ab')2 + 10 nM IL8
10 nM IL8 (Sham F(ab')2 Control)
Branched PEG F(ab')2 Alone
Branched PEG F(ab')2 + 10 nM IL8
10 nM IL8 Branched PEG F(ab')2 Control)
Linear PEG F(ab')2 Alone
Linear PEG F(ab')2 + 10 nM IL8
10 nM IL8 (Linear PEG F(ab')2 Control)
IC50 = 0.5 (All)
% Total Cellular ß-Glucuronidase
Molar Ratio Antibody:IL-8
0.1
1
10
1
2
3
4
5
6
7
8

FIG. 59A



IL8 Alone (F(ab')2 Control)
F(ab')2 + 10 nM IL8
F(ab')2 Alone
IL8 Alone (40K PEG 2 F(ab')2 Control)
40K PEG 2 F(ab')2 + 10 nM IL8
40K PEG 2 F(ab')2 Alone
40K PEG 2 F(ab')2
EC50 = 0.723
Control F(ab')2
EC50 = 0.527
% Total Cellular ß-Glucuronidase Activity
Molar Ratio Antibody:IL8
0.1
1
10
0
2
4
6
8
10

FIG. 59B

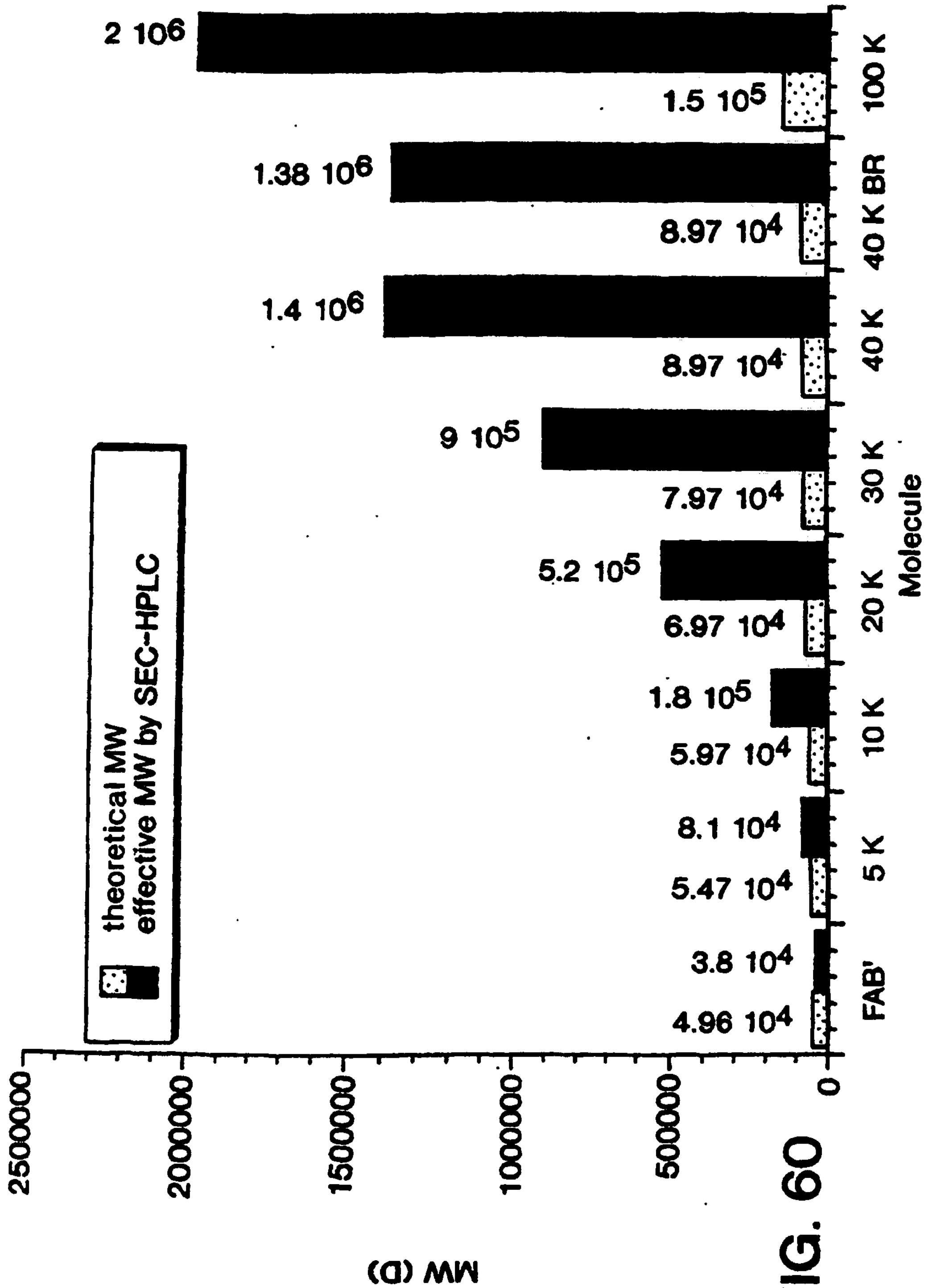

theoretical MW
effective MW by SEC-HPLC
2 10^6
1.5 10^5
1.38 10^6
8.97 10^4
1.4 10^6
8.97 10^4
9 10^5
7.97 10^4
5.2 10^5
6.97 10^4
1.8 10^5
5.97 10^4
8.1 10^4
5.47 10^4
3.8 10^4
4.96 10^4
100 K
40 K BR
40 K
30 K
20 K
10 K
5 K
FAB'
Molecule
2500000
2000000
1500000
1000000
500000
0
MW (D)

FIG. 60

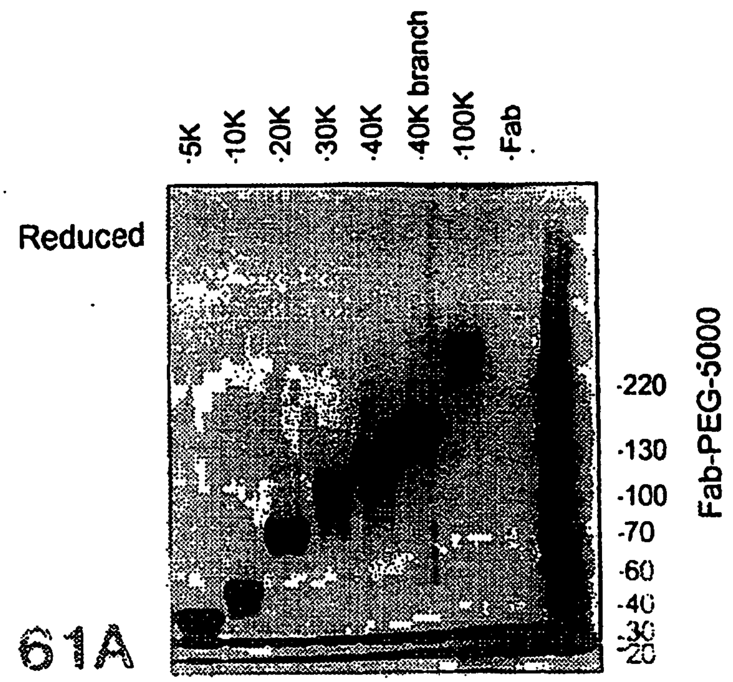


FIG. 61A

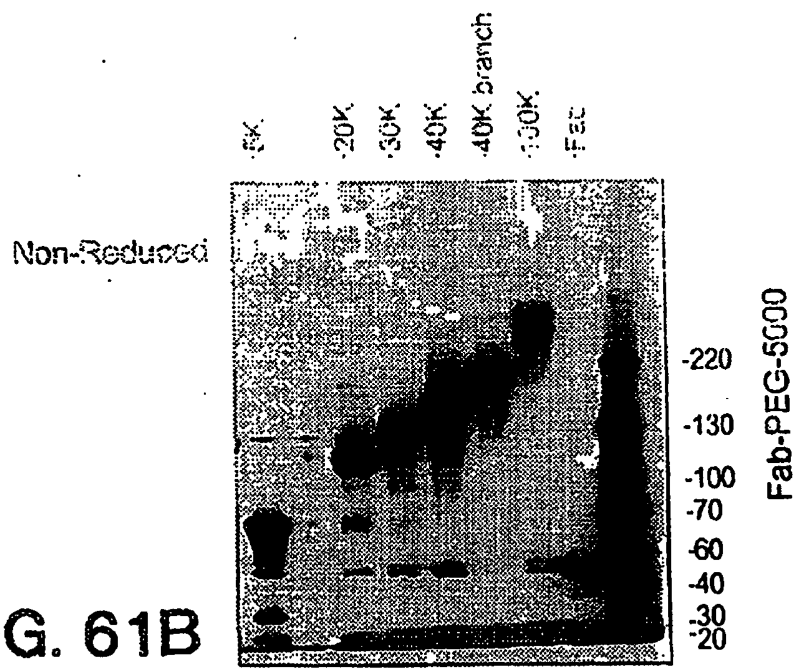
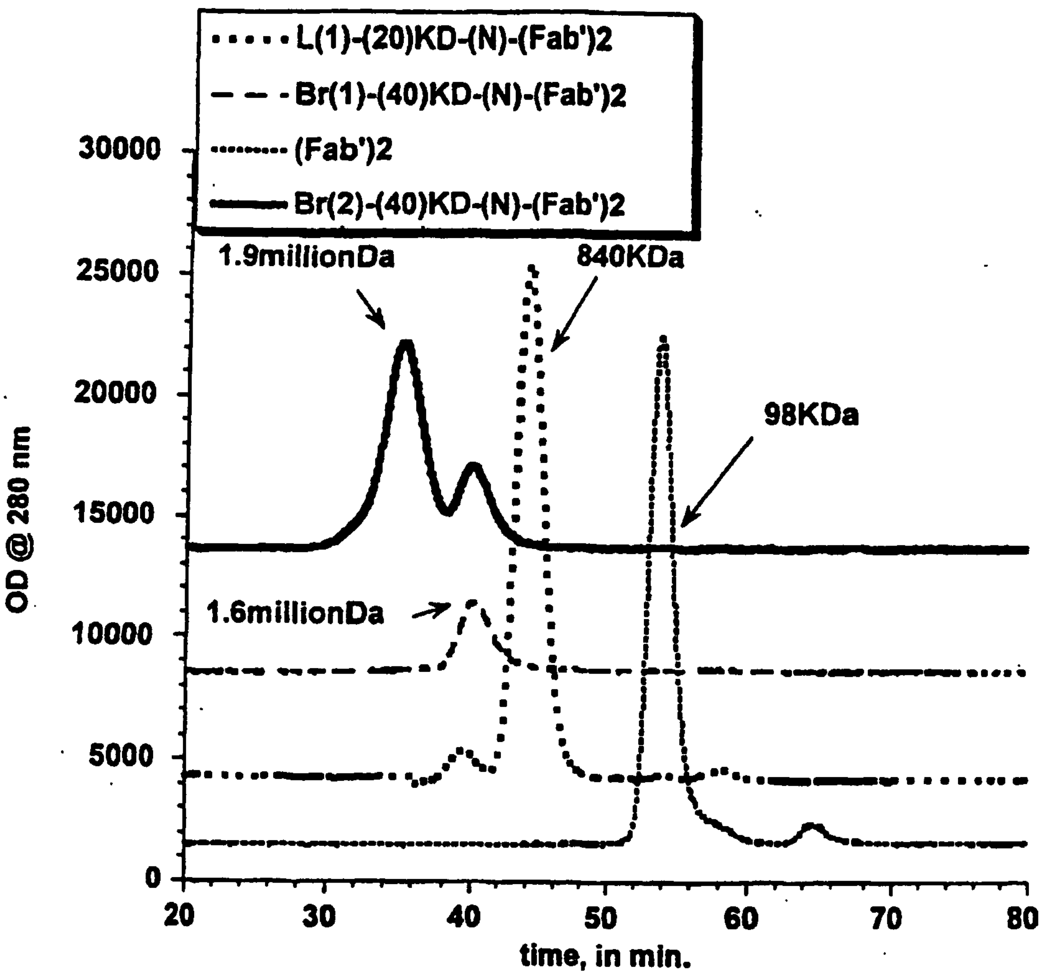


FIG. 61B



L(1)-(20)KD-(N)-(Fab')2
Br(1)-(40)KD-(N)-(Fab')2
(Fab')2
Br(2)-(40)KD-(N)-(Fab')2
1.9millionDa
840KDa
98KDa
1.6millionDa
OD @ 280 nm
30000
25000
20000
15000
10000
5000
0
20
30
40
50
60
70
80
time, in min.

FIG. 62

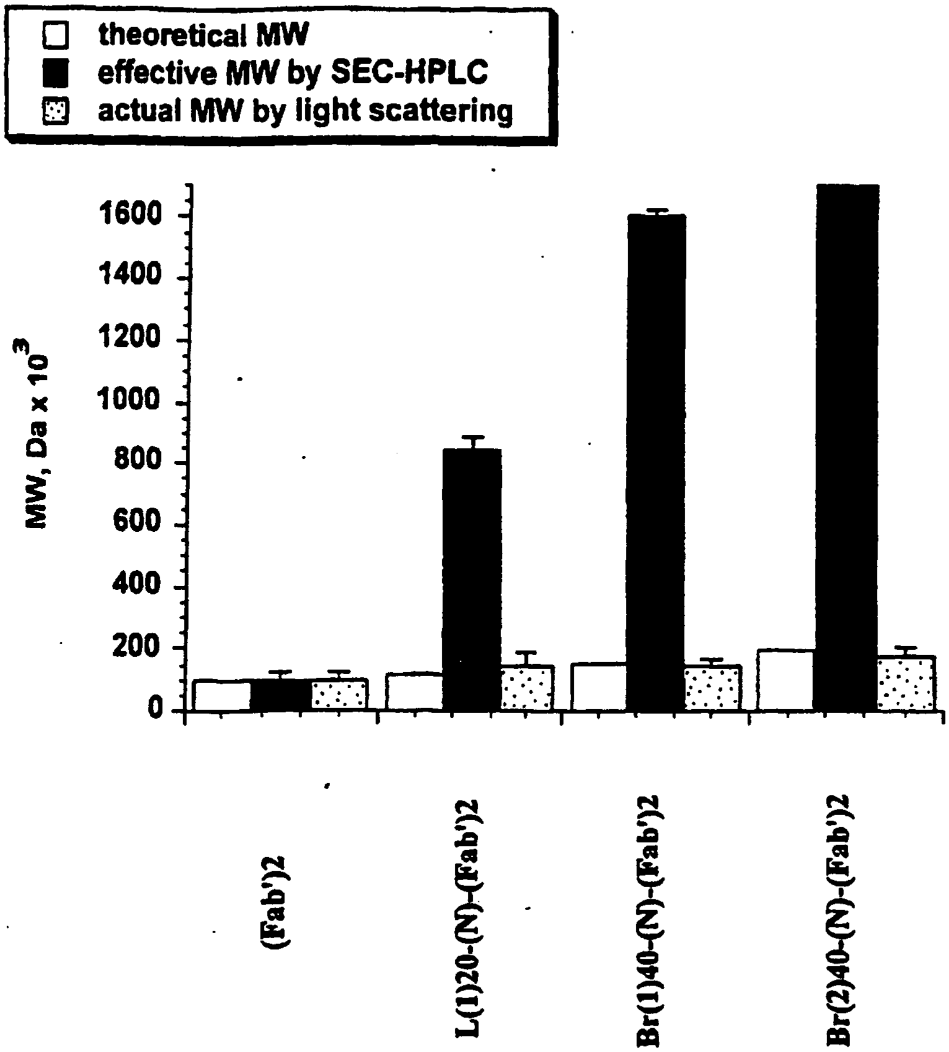

theoretical MW
effective MW by SEC-HPLC
actual MW by light scattering
MW, Da x 10^3
1600
1400
1200
1000
800
600
400
200
0
(Fab')2
L(1)20-(N)-(Fab')2
Br(1)40-(N)-(Fab')2
Br(2)40-(N)-(Fab')2

FIG. 63

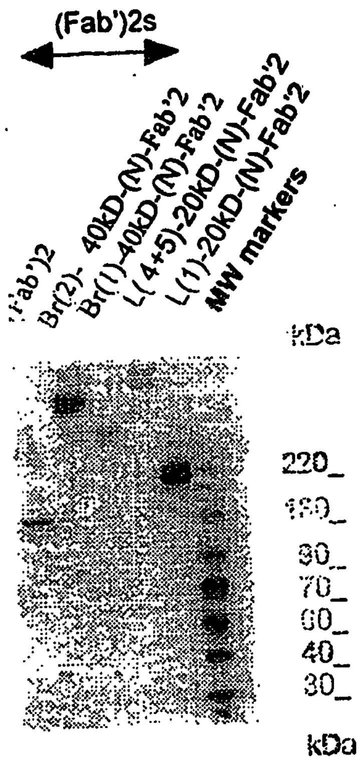

(Fab')2s
Br(2)- 40kD-(N)-Fab'2
Br(1)-40kD-(N)-Fab'2
L(4+5)-20kD-(N)-Fab'2
L(1)-20kD-(N)-Fab'2
MW markers
kDa
220_
70_
40_
30_
kDa

FIG. 64

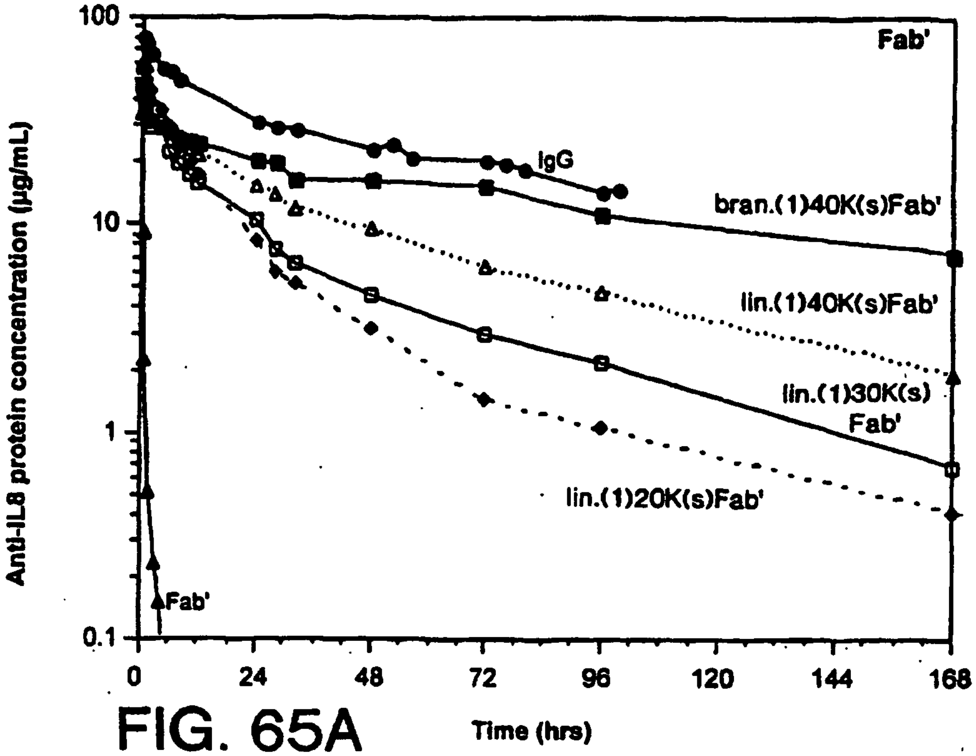


FIG. 65A

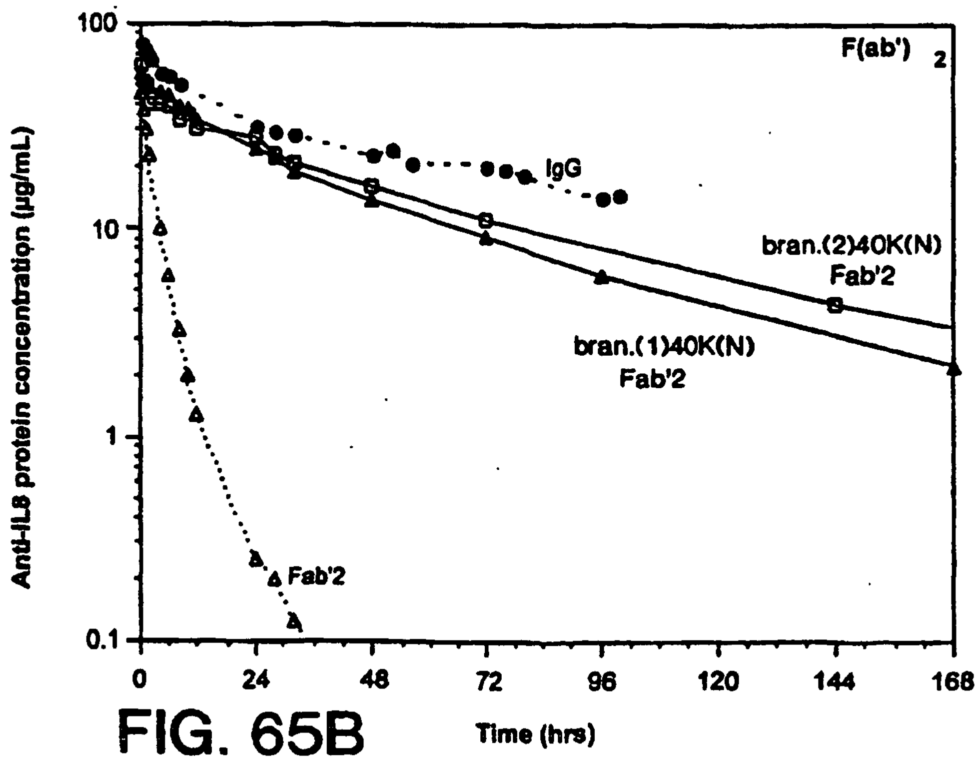


FIG. 65B

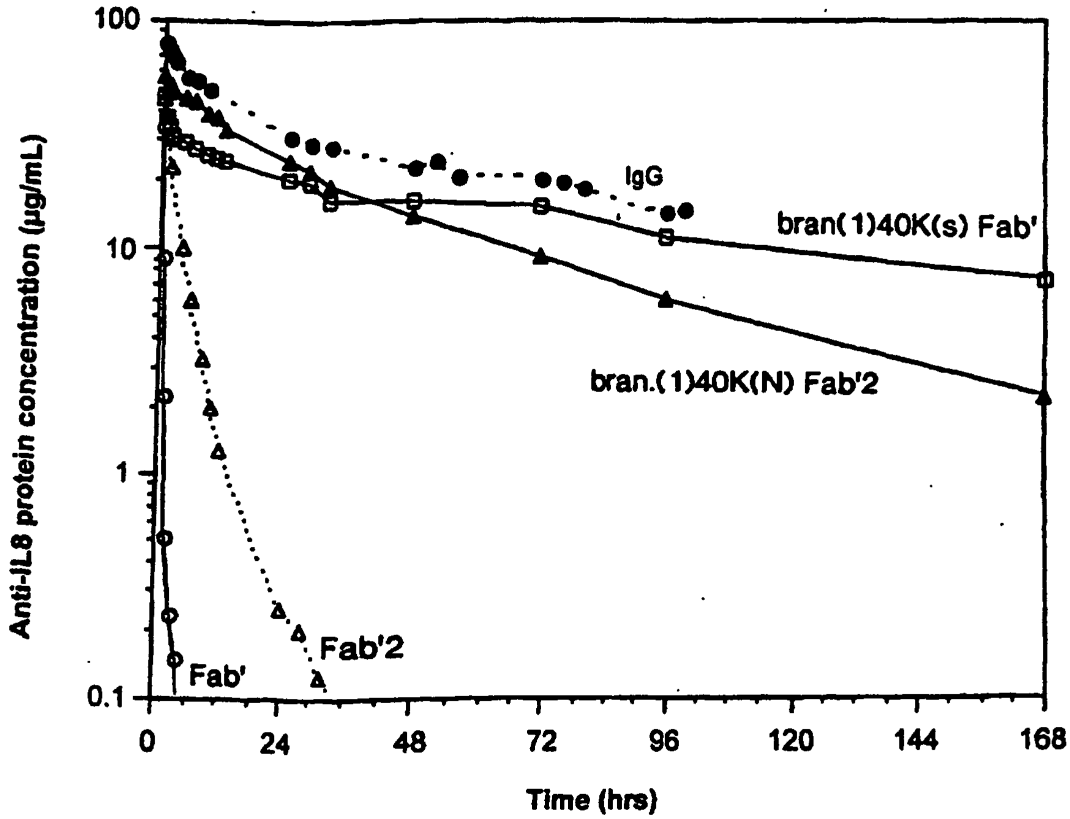

100
10
1
0.1
Anti-IL8 protein concentration (µg/mL)
0
24
48
72
96
120
144
168
Time (hrs)
IgG
bran(1)40K(s) Fab'
bran.(1)40K(N) Fab'2
Fab'2
Fab'

FIG. 66

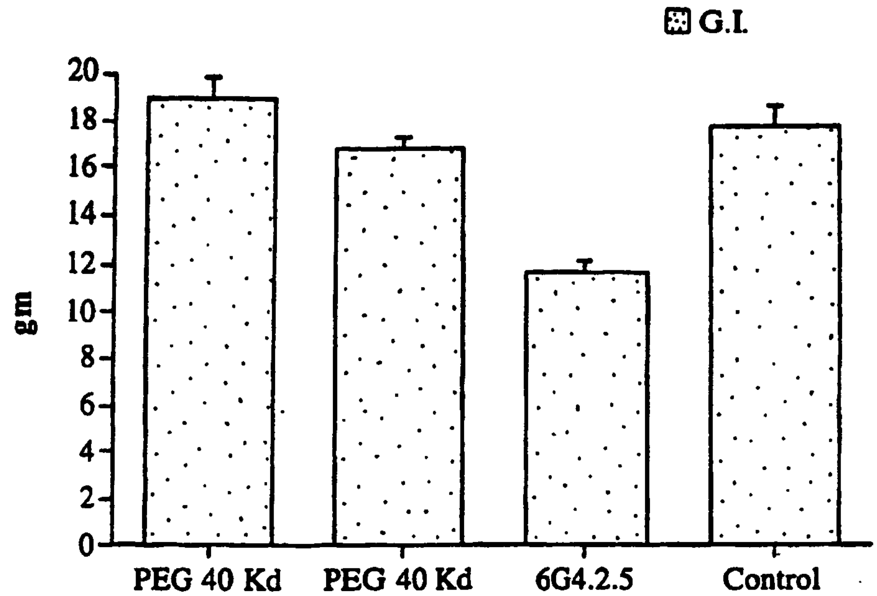

G.I.
gm
20
18
16
14
12
10
8
6
4
2
0
PEG 40 Kd
PEG 40 Kd
6G4.2.5
Control

FIG. 67

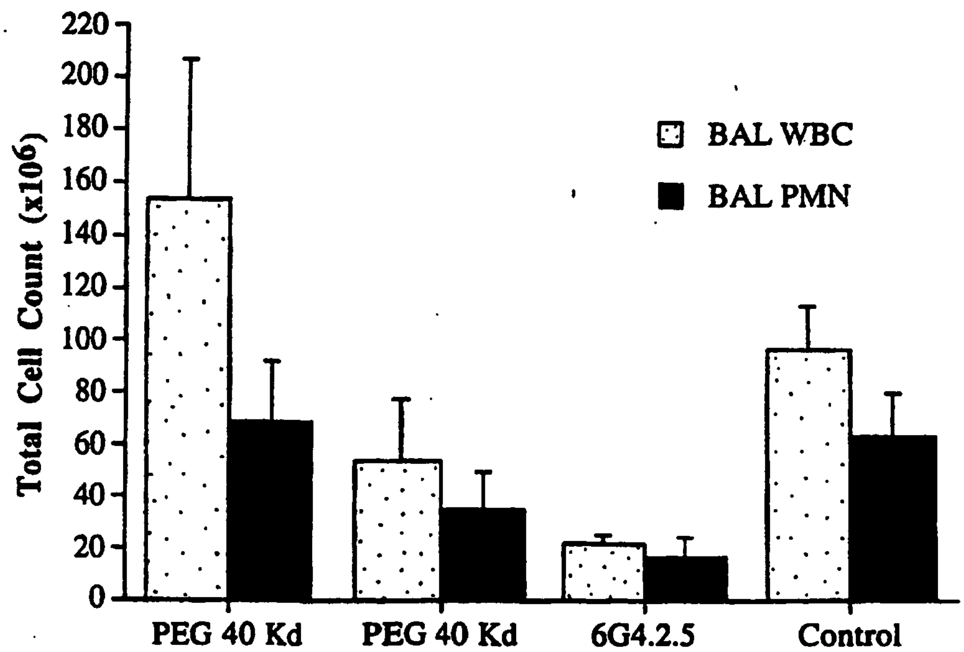

Total Cell Count (x10^6)
220
200
180
160
140
120
100
80
60
40
20
0
BAL WBC
BAL PMN
PEG 40 Kd
PEG 40 Kd
6G4.2.5
Control

FIG. 68

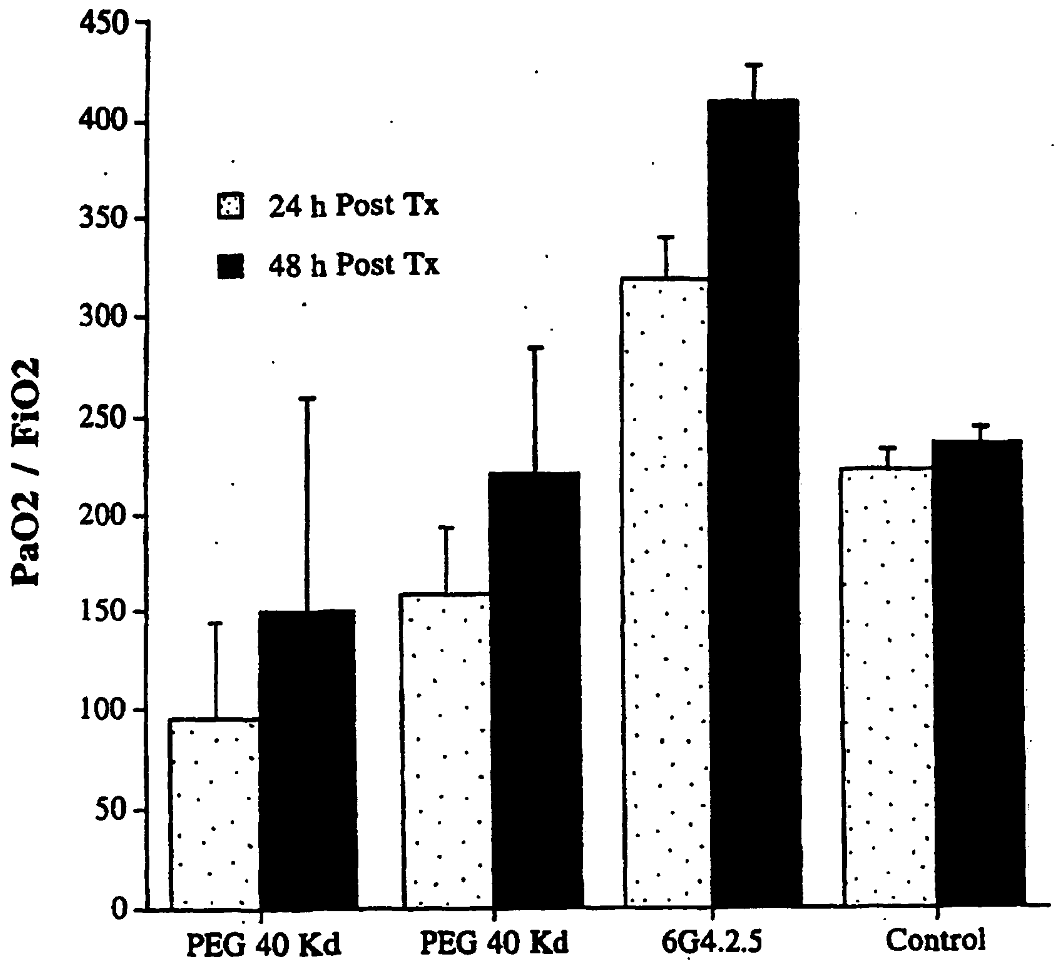

450
400
350
300
250
200
150
100
50
0
PaO2 / FiO2
24 h Post Tx
48 h Post Tx
PEG 40 Kd
PEG 40 Kd
6G4.2.5
Control

FIG. 69